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October 8–12, 1956

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Philip R. White

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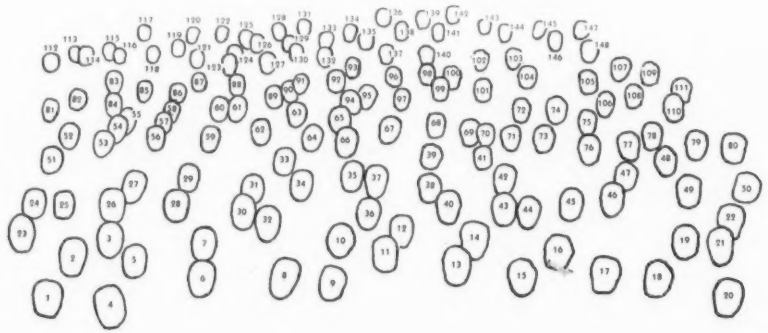

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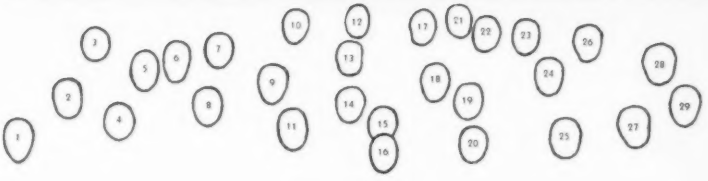

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To the Decennial Conference of the Tissue Culture Association

I send greetings and a welcome to your distinguished scientists from overseas. Your work is basic to the understanding of the cancer problem and to the mystery of life itself. Your imagination, perseverance, meticulous attention to detail give hope that you will soon reach new and beneficial answers to the well being of mankind. Best wishes for the success of your Conference, in the interchange of ideas and cooperative plans for the future.

(Signed) *Dwight D. Eisenhower*

The presence of our colleagues from outside the United States of America has been made possible through the cooperation of:

The Rockefeller Foundation (U.S.A.)
The National Academy of Sciences (U.S.A.)
The Royal Society (England)
The Wellcome Trust (England)
University College, Swansea (Wales)
Direction des Relations Culturelles (France)
Ministère de l'Instruction Publique (Belgium)
Ministry of Education, Arts and Sciences (Netherlands)
Swedish Cancer Society (Sweden)
The Councils of Scientific Research of Italy, Yugoslavia, and India

We wish to express our appreciation for this help.

Participants from the United States and from Canada have their universities, companies, and other organizations to thank for contributions toward their travel. These we trust will accept our appreciation without specific enumeration.

All costs for official participants during the Conference have been paid from funds allocated by the American Cancer Society, acting through the Committee on Growth.

436198—57——2

Albert Fischer

JULY 23, 1891–JULY 31, 1956

In his death

the field of Tissue Culture

has lost one of its founders,

a great investigator

and teacher.

Tissue Culture

Epilogue, Act 1

The principles we seek today
Reside in sap and lymph.

Can tissues stay habitué
In vitro and *ad inf.*?

In spite of toil and tedium
We diligently strive

To find a happy medium
To keep the things alive.

In many ways we culture maize
Or little bits of stick

In sterile tubes in vast arrays
To see what makes them tick.

And many merry tales we tell
Of Haberlandt and Knop

And Madame Fell and old Carrel
And Riker and de Ropp.

Through day and night we burn the light
At temperatures serene;

We put out trust in Philip White
And keep our glassware clean.

So here's to Tissue Culture
We raise our glasses filled

With Gautheret's solution
And water, thrice distilled!

RALPH LEWIN
Yale University,
November 26, 1947

Foreword

The periodic review and reappraisal of any field of human endeavor is a practice whose salutary results are supported by long and oft-repeated experience. This is especially true of one which is developing rapidly. To avoid both fruitless wandering in basically unrewarding cul-de-sacs and the equally undesirable neglect of valuable and unnoted trails, to properly balance the search for new approaches with the development of details in already established ones, to maintain the equilibrium between theory and practice, are not easy functions for any individual by himself. Nor can he always maintain adequate contact with a wide variety of collateral fields without some special mechanisms. Symposiums on a broad base serve these purposes.

Tissue culture (or cell culture), whether one considers it a field, or a point of view, or a method only, has certainly been expanding at an accelerating rate over the past decade and was ripe for such a reappraisal. A Conference on Tissue Culture was called at Hershey, Pennsylvania, in November, 1946, at the instigation of the then young Committee on Growth of the National Research Council. Of the 34 invited participants at that Conference, 21 had at one time or another done some tissue-culture work. The Conference itself brought rather sharply into focus the many problems for which this method offered valid approaches, only a few of which had then received more than the most fleeting attention. It is worth noting that the only mention of viruses was Dr. Coman's discussion of the Shope papilloma! The untapped possibilities were more than evident.

At the end of that session a group of us, led by Dr. Keith Porter, concluded that something must be done in a formal way to hold together those interested in tissue culture, to channel their activities into those directions most likely to help others, and to serve as a center to which other investigators, not then present, might gravitate for future support and stimulation. That group, then designated as the Tissue Culture Commission (later changed to the Tissue Culture Association) consisted of:

Austin M. Brues
Gladys Cameron
Albert Claude
Dale R. Coman
Wilton R. Earle
Honor B. Fell
Albert Fischer
George O. Gey
Ross G. Harrison
Duncan C. Hetherington
Warren H. Lewis
Margaret R. Murray
Richard A. Ormsbee
George H. Paff
Raymond C. Parker
Charles M. Pomerat
Keith R. Porter
Henry S. Simms
J. Vogelaar
Paul A. Weiss
Philip R. White

Journal of the National Cancer Institute, Vol. 19, No. 4, October 1957

During Dr. Porter's term as president the Commission set itself four primary goals. First, in order that workers unfamiliar with the niceties of the method might be adequately trained in its ramifications and might avoid the pitfalls it presented, a training course in tissue-culture methods was to be organized. This became the Tissue Culture Course, first at Toronto under Raymond C. Parker, then at Cooperstown under Keith Porter, John Hanks, Margaret Murray, Don Fawcett, Bill Scherer, and Charles Pomerat, and now at Denver under John Paul. Second, there was to be prepared a complete bibliography of Tissue Culture Literature up to 1950. This proved to be a huge task and under the able management of Margaret Murray was published in 1953 by the Academic Press in two volumes of more than 1,700 pages. Third, in order to relieve the difficulties of small and busy laboratories, which often lacked the complex facilities required, there were to be developed one or more commercial sources of the requisite media, an assignment now ably handled by Difco, Microbiological Associates, Cappel Laboratories, and others. The fourth was to establish and maintain rigorous standards for materials, and for this purpose a testing and certifying service was set up, first under Keith Porter and later under Duncan Hetherington.

The results of this fourfold nursing have been gratifying, to say the least. And as the decade rolled around, the Tissue Culture Association decided that it was time to review what had been accomplished and to try to map out valuable directions in which our efforts should be channeled at the beginning of the next decade. Obviously such a review would involve many more than the 34 participants in 1946. It would be quite different in scope and in cost. We would have to have help.

That help has been forthcoming again from the Committee on Growth, from the Rockefeller Foundation, and from a number of other sources. This volume is the result of the Decennial Conference on Tissue Culture, convened at Woodstock, Vermont, October 7 to 12, 1956. The Tissue Culture Association wishes to extend its thanks to all those who have helped in making it a success.

Philip R. White

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Nutrition and Metabolism of Plant Tissue Cultures[1, 2]

H. E. STREET, *Department of Botany, University College of Swansea, Swansea, Wales*

Mr. chairman, ladies and gentlemen: As the first visiting speaker to this Conference, I should like to express my thanks to the Tissue Culture Association and, particularly, to Dr. White for all the trouble the Association and he have taken to welcome us here. I think, in stressing my personal thanks, I am expressing the feelings of those of us who have come long distances to be with you.

With so large a field of inquiry for consideration at this opening session of the Conference, it is the purpose of the present paper to do no more than set a framework for the ensuing discussions. It should be borne in mind that succeeding sections are devoted to the contributions made by plant tissue and organ cultures to our understanding of morphogenesis and to the special problems raised by the culture of virus-infected and tumor tissues. In taking note of the special fields of research represented by others on the program, it seems appropriate for me to confine myself to the field in which I have firsthand knowledge, that is, the culture of excised roots. One of the most important aspects of this Conference is that problems in different fields should be presented by those actively engaged in their study and in such a way that attention is focused on their possible relevance to others working on different problems or with different living material. In this paper, a quite deliberate choice has therefore been made of certain general problems of nutrition and metabolism encountered in my laboratory, which seem to constitute a suitable framework for elaboration and expansion by discussion.

The nutrition and metabolism of tissue or organ cultures is not something unique and distinct from that of the organisms from which they are derived. Complementary to this, we should not, however, assume that in culture the metabolism of tissues is unchanged from that operative when the same cells are in the environment of the organism. Only thus can we safeguard ourselves from uncritical conclusions regarding the metabolism of plant cultures and unwarranted hypotheses regarding the nutritional interrelationship of the tissues of the plant body.

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[1] Received for publication May 28, 1957.
[2] Presented at the Decennial Tissue Culture Conference at Woodstock Vermont October 8-12, 1956.

The pioneer researches of W. J. Robbins, P. R. White, R. J. Gautheret, and J. Bonner had resulted, by 1940, in the successful growth in completely synthetic media of excised roots of tomato and pea and of certain callus tissues (*1*). This was in marked contrast to the culture media currently used by those working with isolated animal cells and tissues. The elaboration of these synthetic culture media by the botanists formed the basis for regarding the growth requirements of certain excised roots and callus tissues as strictly definable. The danger of this conclusion is that it should be taken to imply that we know the composition of culture media compounded from bottled chemicals. The composition of the completed sterile medium is something that comes into being during its preparation. This preparation necessarily involves the introduction of impurities of unknown composition and chemical reactions between the starting materials. An uncritical attitude toward the composition of culture media is the first step in the development of an approach that ignores the background against which physiological responses are studied. Substances are then endowed with growth-promoting or growth-inhibiting activity without due consideration of how far their effects arise from chemical interactions within the external medium.

This leads to consideration of the importance of culture techniques. It is easy to criticize the pioneers of plant tissue and organ culture on the grounds that they were so intrigued by questions of technique and with the exciting achievement of continuous culture that they made them ends in themselves. Perhaps the pioneers did lack imagination in assessing the applications of their techniques to the study of problems in general physiology, a fault that stubbornly persists. Their preoccupation with technique was, however, a sound reaction to experience. The more interesting questions raised in my laboratory have certainly been posed as a consequence of apparently unpromising studies of problems in technique.

The interest of the pioneers of plant culture in culturability was also not misplaced. Excised root cultures of only a very small number of species have been subjected to intensive study. Our present inability to culture all, or even most, roots (*2*) represents an important challenge only half-heartedly accepted. Recent studies made possible by bringing into culture groundsel roots (*3*) and rye roots (*4*) indicate that the scope of our studies into root metabolism may be greatly enhanced as the problems of culturability are surmounted. They also strongly support the view that intractability to culture arises because of failure to meet special nutritional requirements.

Work with callus cultures has developed on a broader front. Perhaps, too soon in the development of this field, too many different kinds of tissue have been cultured. While many different tissues have been established *in vitro*, detailed studies in their physiology, such as those carried out with excised tomato roots, are lacking. Recent intensive work with the tissue derived from carrot-root phloem is therefore particularly timely (*5*). It seems difficult, from the published work on

callus culture, to estimate how many unsuccessful attempts have been made to culture particular tissues. What is clear is that many tissues have been cultured without sufficient investigation of their nutritional requirements and growth responses to make an assessment of their potential value for physiological research.

The simplest and traditional basis for a general survey, such as this, is to take as headings the major groups of consituents of a typical plant-culture medium. Such a medium contains a solution of macronutrient and micronutrient salts, a utilizable carbohydrate, a mixture of organic growth factors, and also, in certain cases, a complex supplement of uncertain total composition. These groups of constituents can all be regarded as classes of nutrients and are the substances whose metabolism is particularly accessible to study by the plant tissue-culture technique.

Inorganic Nutrition

With excised tomato roots Eltinge and Reed (*6*) obtained symptoms that they attributed to zinc deficiency. The symptoms described were abnormal root-hair development occurring within 24 hours, retardation of growth within 4 days, and early death of the roots. In 1947, Glasstone (*7*) undertook a more comprehensive survey of the micronutrient requirements of excised tomato roots and concluded that the only micronutrient requirements were iron, which had been shown to be essential by White (*8*), and copper. This led her to conclude that the micronutrient requirements of excised roots were simpler than those of the whole plant, "which is not surprising"! These investigations can be taken to illustrate the importance of attention to technique and the danger of concluding too readily that organ and tissue cultures differ fundamentally in metabolism from whole plants.

Eltinge and Reed (*6*) did not give full experimental details and advanced no evidence that excised root growth could be maintained in their medium even in the presence of zinc. They incorporated in their medium 'traces' of titanium, chromium, vanadium, cobalt, nickel, and tungsten. If their traces were equivalent to those adopted by Arnon (*9*), then presumably they added each element at 0.01 p.p.m. At least for cobalt and nickel, we know that these are toxic concentrations. Their level of phosphate was 8 times that of White's medium (*1*), a concentration that is also clearly supraoptimal (*10*). All this suggests that the beneficial effect of the added zinc was an alleviation of a toxicity rather than a demonstration of zinc essentiality. This view is strengthened by the work of Hannay (*11*) who, using a number of purification techniques applied to both the sugar and the inorganic solution, has only succeeded so far in inducing a partial deficiency of zinc.

Glasstone (*7*) used the dithizone test as a check on the freedom of her solutions from contamination. This test gives no reaction with molybdenum. Her purification procedure is also now known (*12*, *13*) to be relatively ineffective for the elimination of molybdenum, the requirement for which is fully met at 0.001 p.p.m. It is not surprising therefore that

further work (*14*, *15*) has shown molybdenum to be essential for the growth of excised tomato roots. A combined method of purification of the sucrose with a cation-exchange resin, and of the macronutrient salts by a coprecipitation with iron oxinate (*15*), has made possible the demonstration of the essentiality not only of molybdenum but also of manganese with both White's medium and the basic medium of Glasstone (*11*). Although convincing demonstrations of the essentiality of zinc and boron have yet to be achieved, the conclusion reached by Glasstone that the micronutrient requirements of excised roots are simpler than those of the whole plant was unjustified and shows every prospect of being disproved.

White (*8*) adopted a micronutrient supplement that included 0.75 p.p.m. potassium iodide and showed that its omission caused a 30 percent fall in the growth rate of excised tomato roots. Now Heller (*16*) in a comprehensive survey of the inorganic requirements of callus cultures has concluded that iodine, together with aluminum and nickel, is to be classified as a "useful" element. Hannay (*11*), re-examining the importance of iodine for the growth of excised tomato roots, has confirmed that its omission significantly depresses growth and leads to early death of a high proportion of the cultures. This iodine requirement could be met by several iodine compounds, including methylene iodide and iodoacetate, but not by chloride. The marked effect of these iodine compounds on excised root growth is of particular interest in view of the uncertain status of iodine as a nutrient for the growth of whole plants (*17*). It may be outside our terms of reference to discuss the concept of "useful" or "beneficial" elements, but with plant cultures we have an opening to clarify the situation.

Despite the unfortunate beginnings to our knowledge of the micronutrient requirements of excised roots, the potential value of plant cultures for studies in this field should not be minimized. Excised root cultures present us with clonal material in a liquid medium that can be prepared from specially purified chemicals. Growth proceeds very rapidly from a small inoculum so that deficiencies can be rapidly induced. The aseptic conditions exclude dust and microorganisms and make possible the feeding of organic substances whose biosynthesis may be controlled by a micronutrient level. Plant material grown under highly standardized environmental and nutritive conditions can be made available for chemical analysis or as a source of enzymes.

The importance of iron availability in plant-culture media is still not fully appreciated. The addition of a suitable concentration of ethylenediaminetetraacetate (EDTA) to root-culture media has not only exposed new physiological problems (*18*, *19*) but dramatically extended the *p*H range over which excised roots can be grown in culture (*20*). Iron availability in White's medium (*1*) becomes critically limiting at *p*H values about 5.2. By using ferric citrate or tartrate, the upper limit of *p*H compatible with iron availability can be raised to *p*H 5.8–6.0. By using 1.0 p.p.m. iron and a suitable molar ratio of EDTA (*21*), iron

availability in a modified White's medium can be maintained and a high level of excised root growth obtained up to at least *p*H 7.2 (*20*).

When the nitrate of White's medium is replaced by an equivalent concentration of ammonium, the medium does not support root growth (*22*). Ammonium has also been reported to be ineffective or unsatisfactory as a nitrogen source for the growth of callus tissues (*23–25*, *16*). By ensuring that iron availability is maintained at neutrality and by buffering the *pH* in this region by an appropriate use of solid buffers, Sheat (*20*) has demonstrated that excised tomato roots can be grown successfully with ammonium as the sole effective source of nitrogen. These advances in technique are not only making possible detailed studies of the conditions controlling the utilization of nitrate and ammonium by excised roots but are increasing the scope of studies in carbohydrate utilization and of the growth responses of excised roots to electrolytes, amino acids, and auxins.

Recently, Heller (*25*, *26*) has successfully grown plant callus supported on ashless filter paper at the surface of a liquid medium. Nevertheless, solid media, prepared with agar or silica gel, always gave consistently better results. This superiority of solid media was attributed to the colloidal nature of the gel. The standard callus-culture media have iron supplied as an inorganic salt and have a *pH* in the range 6 to 8. Under these conditions it is to be expected that iron availability would be a limiting factor for growth. This strongly suggests that the solidifying agent is of importance for callus-tissue growth because it makes possible a contact-exchange phenomenon, similar to that postulated by Chapman (*27*), to explain the ability of plants to use magnetite as a source of iron, provided the root surface was brought into intimate contact with this insoluble iron compound.

Carbohydrate Nutrition

The early investigators expected that tissues derived from chlorophyllous organs would, in culture, possess the faculty of growing without an organic carbon source. Tissue colonies growing *in vitro* and in light have, however, in no case proved carbon autotrophic. Even where the initial explant is rich in chlorophyll, the tissues in culture become very pale green or almost completely depigmented and require an exogenous supply of sugar for growth. Breakdown of chlorophyll synthesis is a classical symptom of iron deficiency, and this aspect of the loss of carbon autotrophy in callus cultures should certainly be investigated.

Gautheret (*28*, *29*) studied the ability of various sugars to meet the carbohydrate requirement of callus tissue derived from carrot-root cambium. Subsequently, the requirements of other tissues have been investigated, although in all cases the carbohydrates have been tested only at single, arbitrary *p*H values and usually at only a single or very limited number of concentrations. Most callus tissues apparently grow best when supplied with sucrose, glucose, or fructose. The marked superiority of sucrose as a carbon source, encountered in the culture of

excised roots of dicotyledons (*3*, *30–33*), does not seem to be paralleled with callus cultures. The present data on the carbohydrate responses of callus cultures show a general similarity to that reported in 1938 by Robbins and Schmidt (*22*) with excised tomato roots. It now seems clear that their finding that glucose was equal or even superior to sucrose as a carbon source for excised tomato roots was determined by their experimental conditions. With the volume of medium per flask, nature of inoculum, and length of culture period adopted, the tomato roots, apparently supplied with sucrose, were growing for the greater part of each experiment in a medium that contained glucose and fructose but was devoid of sucrose and deficient in available iron. If we also take into account the evidence that glucose and fructose can carry significant levels of organically combined iron as impurity (*18*, *34*), the superiority of glucose can be explained if the experiments are conducted under conditions where the form of available carbohydrate is not the limiting factor to growth. To someone used to working with excised root cultures, the rates of growth of callus tissues *in vitro* would certainly suggest that a search should be made for limiting factors among the inorganic nutrients or organic growth factors before decisions are accepted regarding the relative values of different carbon sources.

This point can be illustrated further by reference to the work of Ball (*35*, *36*) with *Sequoia* callus. He explained the improvement in growth of this tissue, resulting from the partial hydrolysis of sucrose during autoclaving, as due to liberation of the more readily utilizable sugar—fructose. Recent work by Ferguson (*37*) has shown that if the sucrose of White's medium (*1*) is sterilized separately by autoclaving in water or by filtration through sintered glass and then added to the remaining constituents sterilized by autoclaving, excised tomato-root growth is often very variable from occasion to occasion and even from flask to flask within one experiment. Some cultures grow almost as well as in normally autoclaved medium; others show a very poor level of growth. The incidence of failures to obtain a medium of normal growth-promoting activity is increased if the sucrose is sterilized dry by treatment with ethyl ether and subsequently incorporated into the medium by solution in cold, sterile water. If the sucrose is purified by alcohol precipitation (*10*) and subsequently ether-sterilized, the medium is consistently without growth-promoting activity. The growth-promoting activity of such a medium can be restored if either 0.05 percent glucose or 0.01 percent fructose is added to the fraction being sterilized by autoclaving. This situation is superficially similar to that described by Ball (*35*, *36*). However, if the monosaccharides are also sterilized without heat, they are ineffective. On the other hand, either gluconic or glucuronic acids at 0.002 percent are effective either added aseptically or autoclaved. Further, if Fe EDTA is substituted for the ferric sulfate of White's medium, then the purified, unheated sucrose is fully effective as a carbon source. This is not a question of the utilization of monosaccharides or of the monosaccharides catalyzing the sucrose-utilizing system but of the dependence

of iron availability in White's medium on the formation from sucrose of traces of chelating acids. Does not a similar situation operate in the callus culture medium?

The outstanding results regarding the carbohydrate nutrition of plant cultures are probably those obtained in work with excised tomato roots. Excised tomato roots demonstrate the superiority of sucrose as a carbon source over all other sugars, sugar alcohols, and organic acids tested (*31*, *32*, *37*, *38*). It is also clear that, in being utilized, sucrose appears to be degraded in such a way that glucose and fructose accumulate in the external medium in quantities stoichiometrically related to the uptake of carbon. Recent studies have also revealed the contrasted growth effects of phloridzin (*38*) and cortical phosphatase distribution (*39*) in seedlings as against excised roots and the importance of phosphate nutrition for sucrose utilization (*38*). These observations have led to the view that the mechanism of utilization involves a sucrose phosphorolysis at the cell surface and the entry into the cells of an organic phosphate (*32*). This hypothesis, although in line with conclusions reached from studies of sugar uptake by other cells and tissues (*40*), requires much further critical investigation. Recent work in our laboratory shows that many sugars, and particularly glucose and fructose, can raise the oxygen uptake of sugar-deficient excised roots to a level similar to that achieved in sucrose. This strongly suggests that the inability of these monosaccharides to support a high level of excised root growth is not connected with their inaccessibility to oxidative degradation.

Excised root cultures have also shown that a number of natural sugars and, particularly, mannose and galactose are inhibitory to root growth. Study of these sugar toxicities has also confirmed and extended the earlier work of Knudson (*41*) on sugar antagonisms (*33*, *37*). The toxicities of mannose and galactose are antagonized by glucose and to a less extent, by xylose. Optimal antagonism is achieved when the glucose: toxic sugar ratio exceeds a critical value, the value being independent of concentration over a limited range and dependent upon the toxic sugar under test. The glucose must be present simultaneously with the toxic sugar, and, although the phenomenon appears to be a case of competitive interaction, the evidence shows that inhibition of the uptake of the toxic sugar is not involved. Galactose, at a concentration that would effect a complete inhibition of excised root growth in 2 percent sucrose medium, will, in short-term experiments, stimulate the oxygen uptake of sugar-deficient roots. We seem to have here a puzzling phenomenon whose further study may well illuminate the general question of the metabolic interrelationships between sugars.

Requirements for Organic Growth Factors

Vitamins

The recognition that excised root cultures have requirements for thiamin, pyridoxine, and niacin made possible the elaboration of synthetic root-culture media prepared from defined constituents (*22*, *42–45*). The

first point to be made regarding these vitamin requirements is that all the evidence suggests that they do not arise from a complete absence from the root cells of biosynthetic ability. An exogenous supply of the vitamin is 'stimulatory' to growth when the cells are only effecting its synthesis at a suboptimal rate; an exogenous supply is 'essential' for growth when the rate of biosynthesis falls below a critical level. If this is the case, then caution must be applied in using data from plant cultures to postulate nutritive interrelationships between tissues within the whole plant. We do not yet know how far the rates of vitamin biosynthesis can be altered by cultural conditions and how far the nutritive conditions in culture are similar to those occurring during whole plant development. The generally held view that callus cultures, in contrast to root cultures, are relatively insensitive to exogenously supplied B vitamins has also not been critically examined.

The second point to be made relates to the specificity of vitamin requirements. As an example, we can take the interesting case of the ability of glycine to replace more or less completely the pyridoxine requirement of the roots of some tomato strains (*46*, *47*). Boll (*48*) confirmed this observation by obtaining full replacement of the pyridoxine requirement of his strains with a mixture of glycine and niacin and full restoration of growth, in the presence of a suboptimal supply of pyridoxine, with glycine alone. Boll (*49*) went on, however, to show that a number of other substances were capable of replacing the pyridoxine requirement of his roots. This group of substances in order of decreasing efficiency comprised: dimethylaminoethanol, ethanolamine, glycine, choline, DL-norvaline, and L-valine. A still larger number of amino acids, although less effective than the substances of this first group, were also capable of partially replacing the growth-promoting effect of pyridoxine when added to a medium containing thiamin as the sole vitamin supplement. In some preliminary bioassay work, Boll showed that some pyridoxine was synthesized by the roots when cultured in a thiamin-supplemented medium. Roots supplied with thiamin plus ethanolamine grew faster and contained a greater total pyridoxine content at the end of the culture period, but their content of pyridoxine per unit of dry weight was less than that of the thiamin-grown roots. These results were considered to support the view that ethanolamine and other pyridoxine substitutes do not act as precursors of pyridoxine but have a sparing action on the limited amount of pyridoxine synthesized by the root cells. The operation of such a sparing action is difficult to visualize in terms of present-day biochemistry. The simpler alternative—that we are not here concerned with an organic growth-factor requirement as such—should perhaps be seriously considered.

Amino Acids

Amino acid stimulations recorded for groundsel root cultures (*3*) and callus cultures (*23*, *50*, *51*) and the much more frequently encountered amino acid toxicities should perhaps also be considered from the standpoint

of their possible effects on micronutrient availability both exogenously and within the tissues. This also underlines the need for a critical study of the phenomenon of amino acid antagonisms elegantly exposed in the recent work of Harris (*52*) on the root growth of aseptically cultured *Avena* embryos and seen in its simplest form in the marked antagonisms between amino acids of similar structure (*e.g.*, phenylalanine and tyrosine, phenylalanine and threonine, glycine and serine, and arginine and lysine).

Gautheret (*5*), reviewing the literature on the effects of complex supplements upon callus-tissue growth, concluded that their activity seemed to depend upon groups of substances rather than on 1 or 2 specific growth-promoting agents. While, in recent work with root cultures, the growth stimulations resulting from additions of yeast extract to White's medium have been shown to be due to specific amino acids (*3*, *4*, *53*), a more complex situation seems to be involved in the response of callus tissues to coconut milk. Coconut milk has been described as causing a dramatic switch-over from a quiescent or mature metabolism to an extremely active meristematic metabolism characterized by active protein synthesis (*54*). The large-scale chemical examinations of coconut milk carried out by Steward and Skoog and their respective coworkers have been based on the concept that this natural nutrient would yield quite new, specific, and highly active growth substances. Single active substances have not only proved difficult to isolate and identify but no one substance or simple combination of substances has fully reproduced the effect of the natural supplement (*55–57*). Paris and Duhamet (*58*) report that the only synthetic mixture which approached coconut milk in its effect on the proliferation of *Scorzonera* crown-gall tissue was one containing 7 vitamins and 11 amino acids.

Steward, Caplin, and Millar (*59*) have tested the growth-promoting effects of coconut milk using a White's medium (*1*) modified by substitution of ferric tartrate (5 mg. per l.) for ferric sulfate and by adjustment of the *p*H to 6.4. Our experience would indicate that such a medium would be of very low growth-promoting activity, and that one factor involved would be its inability to supply iron to the tissues (*18*). How far then is the coconut milk acting as a chelating agent and enhancing cell growth and metabolism by its effect on micronutrient availability in the external solution? Even confirmation that such complex supplements are not active by virtue of their effects on micronutrients in the external medium would of course still leave open the possibility that their intracellular activity is dependent upon their chelating properties.

Auxins

The possibility that auxin action involves intracellular chelation has recently been raised (*60*), but the importance of this concept to the interpretation of the responses of excised roots and callus tissues to exogenous auxins cannot at this stage be assessed. More important, for our present purpose, is the fact that further work will have to be undertaken before a critical assessment can be made of the extent to which the

responses of plant cultures to 2,4-dichlorophenoxyacetic acid (2,4-D), β-indoleacetic acid (IAA), and other auxins depend upon the chelating activity of these substances in the external medium. The best that can be said is that the basic media and *p*H conditions used in recent auxin studies with excised roots indicate that growth in the absence of auxin has in all probability not been limited by micronutrient availability. The chemical mechanisms of the action of auxins will therefore not be discussed in reviewing some recent contributions to our knowledge of the auxin responses of callus and root cultures. It is necessary, however, that future work should be devoted to a careful study of the metal-complexing properties of those molecules that show auxin and antiauxin properties.

The successful establishment of callus cultures has in many instances depended upon the inclusion of IAA or other auxin-like substances in the culture medium (*61–64*). Gautheret (*61*) observed that strains of carrot tissue capable of culture in the absence of IAA gradually altered in their response to this substance. The proliferation of freshly isolated explants was stimulated by additions of IAA at appropriate concentrations; after a period of culture in IAA-containing medium, the tissues no longer responded to the auxin. A similar loss of response to auxin has been reported in other tissues (*62, 65, 66*). It is now clear, also, that certain tissues like that of *Scorzonera*, which, when first put into culture, must be supplied with auxin, can become auxin-autotrophic and fail to show a positive growth response to IAA additions. Gautheret (*5*) has used the term 'anergy' for this change in sensitivity to IAA. Anergized tissue differs from the normal tissue in having a higher content of free auxin (*64*) and in being transparent, friable, and apparently incapable of initiating roots. The anergized tissue also shows more or less pronounced tumor-forming properties (*66–69*). In regard to the evidence that it does not arise by mutation or by selection from within a cell mass of different initial potentialities, Gautheret (*5*) has postulated that anergy is a kind of enzymatic adaptation.

Many unsuccessful attempts have been made to expose and study enzymic adaptation in higher plant cells. The phenomenon of anergy therefore merits more detailed study. It would seem necessary to explore immediately how far anergy is a specific response to IAA and to examine in greater detail and with modern methods the nature of the change in free auxin content reported to accompany the adaptation. It would also seem important to decide unambiguously whether a change in the rate of auxin synthesis or in auxin destruction is involved.

A number of recent investigations have been concerned with the responses of excised root cultures to exogenous auxins and antiauxins. One aspect of this work arose from the realization (*18, 70*) that excised tomato roots can be maintained in continuous culture in White's medium only by repeatedly choosing newly initiated lateral meristems for subculture. When the main axis tips of tomato roots are excised at weekly intervals, their meristems remain active for only a limited period; with

time, the number of functional meristems gradually declines and ultimately all meristems cease growth (*71*). Similarly, when excised tomato roots are grown without subculture in continuously renewed medium, the main axis meristems cease growth while the more recently initiated lateral meristems are still growing actively (*72*). The duration of activity of the main axis tips is prolonged by the repeated-subculture procedure which, presumably, interrupts and decreases the intensity of the influence on meristematic activity of the developing root system; and this prolongation could be enhanced by more frequent subculture. The survival of main axis meristems, either repeatedly subcultured or on roots developing without subculture in constantly renewed medium, was also enhanced by low intensity illumination of the cultures (*73*), lowering the sucrose concentration (*71*), or by appropriate concentrations of the antiauxins, α-(1-naphthylmethylsulfide)propionic acid (NMSP) or 1-naphthoxyacetic acid (1-NOA) (*19*, *74*). The antiauxin concentrations required to effect optimal stimulation of meristematic activity increased parallel with the extent of meristem 'ageing'; supraoptimal antiauxin concentrations that caused growth inhibition during the treatment passage led to growth stimulation on subculture of the meristems into standard medium. Survival of meristems was reduced by treatment with IAA (*19*) and 1-naphthalene acetic acid (NAA) (*74*) but not with β-indolylacetonitrile (IAN). These auxins were without a significant effect in single-passage experiments when used at very low concentrations (*e.g.*, 10^{-10} gm. per ml. IAA, or lower); at higher concentrations they were inhibitory (*75*). NAA was of particular interest in that, during successive subculture, concentrations initially without significant effect became inhibitory as the meristems 'aged,' and it not only increased the number of meristems ceasing activity at each subculture (the characteristic effect of IAA) but also reduced significantly the initial period of 100 percent survival. The effective antiauxins showed marked antagonisms with NAA but not with IAA (*74*).

Study of the subsequent growth of lateral root tips excised at intervals after their emergence showed that young meristems subjected to repeated subculture go through several passages during which the growth rate rises progressively before this gives place to the phase of decline in growth rate and onset of loss of activity. Older lateral root tips have a higher initial growth rate, which more quickly reaches its optimal level and gives place to the phase of declining growth rate and survival.

These observations suggest that the activity of root meristems is controlled by an essential growth hormone which initially is present in suboptimal amounts but which, in tomato roots grown in White's medium, gradually accumulates to reach a supraoptimal level, where it first depresses growth and ultimately, at a critical concentration, destroys the activity of the meristems. The concentration of this hormone at the meristem seems to be controlled by the organic connection between meristem and root system, and to determine the degree of apical dominance by its control on meristematic activity. Careful analysis of the

effects of IAA on growth and survival of meristematic activity strongly suggests that it is not identical with the natural auxin involved. This has prompted the chromatographic examination of the growth substances extractable from excised tomato roots. Preliminary work (*76*) has already revealed the presence of 2 water-soluble fractions present at much greater activity than that of the free IAA and IAN. It may be that further study of the changes in absolute activity and the balance between these water-soluble hormones and the free IAA and IAN of the root tissues, occurring during the 'ageing' of excised tomato roots, will shed new light on the hormonal control of root growth and be of relevance to the general problem of ageing in plants.

The auxin situation in excised tomato roots seems to be paralleled by recent (unpublished) observations by J. R. O. Dawson working in our laboratory with roots of red clover varieties. There is, however, strong evidence that the excised roots of other species develop, in culture, suboptimal rather than supraoptimal auxin levels. The successful culture of excised rye roots (*4*) was made possible by supplementing White's medium with yeast extract. The yeast extract owed its activity to its tryptophan content and the tryptophan was replaceable as an essential growth factor by IAA. Naylor and Rappaport (*77*) probably encountered another case of absolute requirement when they succeeded for the first time in culturing excised roots of the pea variety "World's Record" by adding to the basic medium a mixture of tryptophan and cystine (they apparently did not test these amino acids individually).

In other cases, although successful culture has not been dependent upon an exogenous supply of auxin, IAA, at appropriate concentrations, has been found to stimulate significantly excised root growth (*78–82*). Evidence that root cultures may contain very low concentrations of auxins also comes from auxin extraction studies, which have been carried out with excised roots of *Helianthus*, maize, and pea (*83–86*). Some of these roots, which show growth stimulation in response to IAA and which appear to have a very low concentration of natural auxin, have been subjected to repeated subculture by excision of their main axis tips. They do not show the 'ageing' phenomenon encountered with tomato roots. Some of these roots (*e.g.*, pea roots) are also characterized by a paucity of laterals and therefore cannot readily be established as clones (*87*). Light, which retards 'ageing' in tomato, also retards lateral initiation in pea roots (*88*).

The concept that root cultures of a number of species are limited in growth and development by the suboptimal level of natural auxin developed in the cultured tissues is also supported by some recent work of Charles (*53*) with roots derived from geographic strains of groundsel. The roots of different geographic strains of groundsel show marked differences in growth habit and growth rate when cultured in a White's medium supplemented with arginine (*89*). Some strains like Iceland grow rapidly, develop vigorous laterals, and can be readily established as clones. Others like the Czechoslovakian, Wetherley, and Manchester

strains grow very much less actively but can, with difficulty, be maintained in continuous culture. Others like the strain from Limoges, grow so poorly that clones cannot be maintained. Charles (*53*), starting with a detailed study of the nutritive requirements of the Czechoslovakian strain, showed that the effect of yeast extract could not be fully reproduced by supplying either arginine or lysine. Yeast extract in contrast to these basic amino acids stimulated lateral initiation. More interesting was the observation that roots grown for 1 culture period in yeast-supplemented medium showed, during a subsequent passage in arginine-supplemented medium, a marked improvement in both linear growth of main axis and laterals and in number of laterals per root as compared with roots in their second passage in the arginine medium. The effect of yeast extract could be reproduced by appropriate concentrations of either tryptophan or IAA. 2-Naphthoxyacetic acid (2-NOA) effected even more marked growth stimulation. At appropriate concentration, this auxin (*a*) during the treatment passage not only increased lateral number but also markedly stimulated main axis growth and (*b*) in the subsequent passage in arginine medium indirectly caused greater growth stimulation than any other treatment tested. It gave roots of healthy appearance with abundant laterals developed not only in the inoculum portion of the root, which had been subjected to the direct action of the hormone (this was characteristically the case with tryptophan and IAA) but also on the new growth.

Charles then went on to work with 4 geographic strains that, when cultured in arginine-supplemented medium, showed progressively lower growth rates in the following order: Iceland, Czechoslovakian, Peruvian, and Wetherley. All these strains were stimulated to varying extents during the direct treatment with 2-NOA, but, by using in each case an appropriate concentration of the acid, all the strains could be raised to a similar optimum and very high level of growth during the ensuing passage in arginine medium. Further, the slower growing strains required a higher treatment concentration of 2-NOA to reach the optimal growth rate in the arginine passage. These strains, with the exception of the Wetherley strain, also required higher concentrations to cause inhibition and irreversible inactivation of the meristems. Detailed study of these results strongly suggests that the roots from different strains differ in growth rate and morphology because of their different suboptimal contents of a natural growth substance and the appropriate concentration of 2-NOA can make good this deficiency. When this work is considered against the background of the study of the inheritance of excised root growth rates in these groundsel strains, undertaken by Skinner (*89,90*), a way into the physiological genetics of excised root cultures begins to take shape.

Conclusions

It should be emphasized that no attempt at comprehensive treatment of a decade of work on the nutrition and metabolism of plant tissue and

organ cultures has been attempted. Further, in setting out below some general conclusions, license will be taken to introduce certain questions relevant to this section, which have not been previously referred to or only very inadequately treated.

As a basis for discussion and as a summary of the author's views on the status of knowledge in this field, the following conclusions are advanced:

1) Quite inadequate attention has been given to ensuring that the inorganic nutrients of sterile plant-culture media are presented in adequate but nontoxic concentrations and in available form.

Interactions between the inorganic constituents and between these and the organic constituents requires detailed study if incorrect conclusions are not to be drawn regarding the photosynthetic ability of cultured tissues and their ability to utilize different sources of carbon and energy.

If the growth effects of electrolytes and the activity of surface-localized enzymes and carrier molecules are to be studied, then tissue and root-culture media will have to be elaborated which present the inorganic nutrients in an available form over an adequate range of physiological *p*H. A number of 'classical' conclusions regarding the growth requirements of plant cultures must be regarded as requiring confirmation in view of the uncertain status of ion availability in the media used.

2) Some progress has been made in defining the micronutrient requirements of tissue and organ cultures but the potentialities for critical studies in this field have yet to be exploited.

3) The early and important additions to our knowledge of the biosynthesis of the vitamins and of their specificity, made in pioneer studies with excised roots, have not been followed up actively during the last decade.

4) The problems in carbohydrate metabolism raised in work with plant cultures can now be studied to advantage only if the techniques of aseptic culture are combined with those of biochemistry, particularly of enzymology.

5) Organ and tissue cultures are uniquely suited to studies of the hormonal control of meristematic activity. Further progress waits upon new knowledge of the natural growth hormones and of the influence of cultural treatments on the balance, within the cultured tissues, of these natural growth regulators.

6) It is not yet possible to make any critical assessment of the importance of physical factors, such as temperature, light, oxygen, carbon dioxide tensions, and colloidal properties of media, in controlling the growth and development of plant cultures. The past decade has shown a growing awareness of the necessity to control and specify such factors.

7) Recent advances in the techniques of media preparation and of clonal establishment warrant renewed attempts to bring into culture callus tissues and excised roots, from those species and varieties within species, which are so far refractory. Progress in this aspect of plant culture may be expected to yield material particularly suited for special studies in plant physiology. The resulting development of our knowledge

of comparative physiology should open up particular aspects of physiology to genetical analysis.

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DISCUSSION

Dr. Steward: May I draw attention to a few points that might be made about Dr. Street's remarks:

First of all, we must remember that a root is a root and not merely a bunch of cells. In other words its nutritional requirements are quite complex. It consists of definite regions, which I am sure we shall read about in the sections on morphogenesis. Different parts of the root are doing quite different things. Their metabolic properties supplement each other. In fact, even in the root apex, particularly in certain roots that I am very familiar with, namely, the root apex of mint, there are little pockets of large, apparently quiescent cells. Obviously they have some part in the whole organization and function as the quiescent region or the cytogenerative region as described by Clowes. I merely point out, that because requirements for some roots may seem simple, it doesn't necessarily follow, from the organizational standpoint, that the requirements for each individual cell in that root are also simple. It is for this reason that we have tried to tackle the problem by finding out what the requirements are for individual cells to grow, *i.e.*, to divide and to elongate. I will leave the problem of differentiation and development for others or for a later time.

Professor Street has made quite a good deal of this point about trace elements, quite rightly. One of the first things of which we tried to assure ourselves was that when we needed to fractionate enormous amounts of organic material in search of growth factors, we weren't merely chasing molybdenum, manganese, iron, or something of the sort. In fact, I come from a department where one of my emeritus colleagues made some of the first contributions to the effects of organic matter on the availability of trace elements. We can take these materials, critically extract them with dithiocarbazone and other agents to get rid of trace elements, and add back trace elements, and we still find evidence of dramatic effects of the *organic* materials in these complex media. There is, however, one rather simple experiment that illustrates the extreme specificity of these effects. Potato cells will not grow continuously on any sort of medium I know of, whether you put in coconut milk, or corn extract, or *Aesculus* extract, or what not. But if you put in synergistic mixtures of 2,4-dichlorophenoxyacetic acid (2,4-D) or some other substances in very small amounts, along with the natural extract, these cells can be made to grow very rapidly. Moreover, if you use a synergist with an optically active carbon atom like an α-substituted propionic acid, the D-isomer is active, the other one, L, is inactive. Not only that, but the L-isomer will completely counteract the effect of the D-isomer if you increase its concentration. By this treatment you have not disturbed the organic complement of the medium in respect to vitamins, coconut milk, trace elements, etc., and the resultant effects are merely the difference between the addition of pure D-α-naphthoxypropionic acid or its L-enantiomorph. I find it very hard to explain facts of this sort entirely by the effect upon availability of trace elements.

Professor Street seems to feel that because the requirements of certain root cultures are simple, the requirements of all other cultures must be simple too. I take the view, very definitely, that we know all too little about the nutritional requirements of individual tissues. In fact I go further, for if we really knew what was necessary to start some of these cells to grow in the way that we are now studying them, we would then need to go back and redesign the inorganic medium completely in order to specify what might be called the best nutrient requirements. We have concentrated on what we thought was the first task, namely, to find the requirements for maximum growth. Now, for example, let me illustrate.

Although we leave the calcium content more or less where it is, in Dr. White's basal medium, we know that, at least for carrot cultures growing in presence of coconut

milk, this is far in excess of what is needed. If you take a medium without any calcium and remove the calcium from coconut milk with oxalate, it is astonishing how well these tissues grow. In fact, if you then add back varied calcium and potassium concentrations to find the optimum, you observe that once the tissues have started to grow in the presence of the growth factors, they will keep on growing in a *very* low concentration of calcium. They behave more like algae or fungi in a solution in which carrot roots and whole plants would find it very difficult to survive. These effects I cannot attribute to chelation, which has been emphasized and which, incidentally, we have considered too.

Reference has also been made to the role of casein hydrolysate, which is a convenient way of satisfying the requirements of the tissue for reduced nitrogen compounds, but we are quite clear that in the presence of the coconut milk even ammonia would very largely suffice. This use of casein hydrolysate is merely a device to avoid trying to specify all the complexities of the amino acids before we understand the complexities of the growth factors. Probably I had better end here by saying that we would agree with Dr. Gautheret or anybody else who says that the coconut milk, *Aesculus* extract, corn extracts, etc., contain complex mixtures of substances. We believe that there is no single substance that unlocks the door of growth by cell division. There are very many substances, natural and artificial, that will do this in greater or lesser degree. The problem, as we see it, is really to find out what these substances are and then, by their aid, having unleashed the potentiality for maximum growth of the cells to study the finer points of their nutrition. One would hardly study the trace-element requirement of an autotrophic plant without insuring that it had maximum availability of CO_2 for organic nutrition. It is obvious that we really need both nutrients and cell-division stimuli. We have tried to do what we thought were first things first, namely, to isolate and, where possible, identify the cell-division factors and then study the nutrient effects later.

For reasons of time, the following points, relevant to the program as a whole, could not be developed but are here mentioned in summary only.

1) In focusing attention upon the twin problems of growth of tissue cultures by cell division and cell enlargement, it has been necessary to develop techniques, based on maceration and photography of representative hemocytometer fields, by which cell number and hence average weight per cell can be determined. In this way we may speak not merely of growth but of the quantitative contribution to growth of the cell division and the cell enlargement.

2) In the attempt to approach even more closely the cellular basis for tissue cultures, methods have been developed in our laboratory by which cultures have been maintained continuously for over 2 years from inoculations made from suspensions of free-floating carrot and potato cells, and the way in which these cells grow and organize into more conventional tissue cultures has been described.

3) There are many metabolic contrasts that may be drawn between the rapidly dividing tissue and that which is either not dividing at all or only doing so very slowly. This is shown by the different amount and relative amino acid composition of the alcohol-soluble nitrogen in the tissues and by the almost universal presence of hydroxyproline in the protein synthesized in the dividing cells.

The growth induction is also accompanied by changes in the behavior of the tissue toward enzyme inhibitors and radiation. Whereas the tissue prior to growth induction is so sensitive to carbon monoxide and radiation that its ability to respond subsequently to coconut milk is easily obliterated, the tissue in which growth is already induced is less sensitive to carbon monoxide and radiation but more sensitive to nitrophenols.

4) The molecules that are responsible in nature for the type of growth that we have studied in carrot cultures are very varied. Compounds A, B, C, and F from coconut milk are all different but separately active in presence of casein hydrolysate, though with variations in their relative activity from carrot root to root. Only Compound A is identified (*1,3*-diphenylurea).

Both in coconut milk and other extracts (*Aesculus*), compounds belonging to the

leucoanthocyanins occur and are found to be active in promoting cell division. Many related substances are also effective to some degree.

Kinetin, in presence of indolacetic acid, has some effect, more on carrot tissue than on artichoke, but its effects are small compared with whole coconut milk.

Gibberellic acid, contrary to expectations, did not stimulate cell enlargement *per se* in our cultures, though it did produce some effects on cell division.

Dr. Nickell: I would like to comment on 3 points that Dr. Street mentioned. First, I am in complete agreement that the past preoccupation with technique has been worthwhile. At least in the plant side of the field, every time we move forward and stop, it is strictly a hurdle of technique which must be overcome to move on. This was certainly apparent in working with Monocots. I noticed in the discussion the general observation that liquid media were not as good as solid. Actually, if one can use the correct techniques, one can increase growth tremendously in liquid media over solid media. I am sure this will be discussed later. It is just another example of the correct use of existing techniques or the use of new techniques.

The second point is the quantitative role of iron. In view of the more recent work with antagonistic toxicity of some of the other metals with iron and also the still undetermined role of chelation, we have stopped to reconsider what we previously thought was the qualitative role of iron. The knowledge of the role of chelation in nutrition and metabolism certainly is still in its infancy. Dr. Street has commented concerning the fact that the use of a synthetic medium does not mean we know what the medium contains but only that we know what we put in it. We found, several years ago, that certain antibiotics increase the growth of various plants and certain plant parts. We also found that if these compounds were put into solution and permitted to degrade, the degradation products were much more effective and probably were the key compounds in causing this growth effect. Fortunately, immediately prior to this physiological work, the tremendous job of determining the structure of these extremely complex molecules had just been finished and hundreds of intermediates were available for us to test. We have been for the past several years, and still are, very actively testing these compounds and finding some almost fantastic types of molecules. All that I can say is that the ones which seem to be most effective in regulating growth are divided into 2 groups. Some seem to fall into a group having chelating properties. The second group are those that apparently cause the growth effect as growth factors and not because of their ability to chelate.

Third, Dr. Street's statement concerning the possibility that when a vitamin is apparently required, it may not necessarily be nonexistent in the plant part, particularly in the roots in which it is working. This brings to mind some work that we did some time ago. The *Rumex* tissue with which we worked, which was from a root, required thiamin. Its absence caused death very quickly. Certain compounds, when put into the medium, apparently spared thiamin yet in no conceivable way could they supplant it or serve as a substitute. This fits in with Dr. Street's idea that it is quite possible that thiamin might be being manufactured in the root but at such a low level that without an exogenous supply the root would die; yet addition of certain types of compounds might open up this synthetic pathway, at least for a time, so that the root could produce the thiamin it requires. Quite possibly, in the entire plant there is a regulatory mechanism by which the roots, which we consider to require such compounds, actually are enabled to produce them themselves.

Dr. Riker: The unusual experimental facilities available to those working with plant tissue cultures are perhaps worth listing. First, the plant tissues grow on media containing only nutrients for which we know the chemical formulae, *i.e.*, on synthetic media. Second, we can incubate the tissue transplants for a month to 6 weeks without further attention. This is a great saving of materials, and it frees the technicians for other tasks. Third, these tissues grow in a relatively compact mass without dendrites or pseudopods. To measure the amount of growth, we remove the tissues from the medium and take either wet or dry weights.

Either agar or liquid media may be employed. The agar is usually about 0.6 percent. The liquid media usually require abundant aeration if the growth is to approach

that on the surface of agar. For chemical analysis of metabolic products, the liquid medium is advantageous.

Such experimental possibilities make available a wealth of well-established bacteriological procedures for work with tissue cultures of higher plants. These have been employed by Doctors Black, Braun, Gautheret, Morel, Nickell, White, and others.

The bacteriological techniques offered so much promise that Dr. Hildebrandt and I undertook to employ them for measuring the physical and chemical requirements for the growth of the tissues. After determining the best temperature, hydrogen-ion concentration, balance of mineral salts, growth substances, and so on, we began on some important common sources of nitrogen and carbon.

Tissues were used that usually came from crown gall, because (*a*) healthy tissues often required a supplement found in coconut milk and (*b*) to carry both "diseased" and "healthy" tissue before critical points were clarified doubled the amount of work.

Several complicating factors have appeared: (*a*) Since certain bacteria and fungi remain alive in some tissues, a frequent check on their presence has been necessary. (*b*) The commonly recommended amounts of nutrient, *e.g.*, 1 percent, were sometimes inadequate. We found it necessary to make serial dilutions, usuall · too much, half of that, half of that . . . until too little. The amount was often as critical as the substance itself. (*c*) Different kinds of tissue sometimes responded differently—one tissue's nutrient was another tissue's poison. (*d*) The same thing applied to inhibition of growth by common metabolites. Some simple amino and organic acids unexpectedly slowed or prevented growth. (*e*) The size of the tissue transplant often was important. Large transplants often grew when small ones failed. Single cells were especially fastidious. (*f*) The age of the tissue since the previous transplant sometimes influenced the lag period before active growth began. Any glib generalities that fail to consider such factors may lead to misinterpretation.

The isolation and growth of single cells through the development of "nurse" techniques by Doctors Muir, Hildebrandt, and myself have been particularly interesting. Dr. Steward already has pointed out the different kinds of tissues in root tips. Numerous clones (Dr. Earle's definition) all grown from single cells have been established from callus cultures. Clones, which were all derived from a single mass of callus, have varied quite a bit in a number of characteristics. We suspect that pathological growths arising from crown gall may be a mixture of gall, intermediate, and healthy cells with differing growth potentialities.

These studies have suggested that the balance between different nutrients and metabolic products might be critical for determining whether a tissue was able to grow, whether it was inhibited from growing, or whether it differentiated in one direction or another.

Dr. Gautheret: I have always been surprised that the nutritional requirements of roots appear to be different from those of true tissue cultures. It is sometimes possible to obtain a callus culture from a root culture and the reverse can also be obtained; that is, a callus culture may give rise to roots. The genetics of the cells should be the same, yet there are changes in the nutritional requirements. Callus cultures may need indoleacetic acid or another auxin and may not require vitamin B_1, while the root cultures may need vitamin B_1 and not require auxin. This is surprising. I am sure it is not necessary to give you other examples. I feel that the question of nutritional requirements is not a specific property of the cell itself. These differing requirements may be related to the phenomenon of organization among histogenetic processes.

Some callus cultures can be grown on mineral salts and sugar alone and don't require any growth factors. This is the case with the carrot tissue, blackberry tissues, and most of the tumor tissues such as crown gall. Other tissues require the same basic nutrients with the addition of an auxin. This is the case with the tissues of Jerusalem artichoke and Virginia creeper. Others require vitamin B_1 such as the virus tumor tissue of *Rumex* studied by Black and Nickell and the normal tissue of Hawthorn (Mlle. Paris). In these tissues vitamin B_1 is as necessary as auxin for Jerusalem

artichoke tissue. Many tissues don't *require* auxin or vitamin B_1, although one can observe strong stimulation with one or the other of these growth factors.

Now, let us consider the very interesting natural extracts studied extensively by Steward and his collaborators. It is true that coconut milk has a very powerful effect on the growth of callus cultures and that no synthetic substance has such an action, but when one tries to determine the components that promote this action the question becomes very difficult. When you isolate any one of these components, its activity proves to be much lower than that of crude coconut milk. There are more than 15 such substances, which have been isolated and which have analogous activities. Professor Street spoke about the mixture devised by Mlle. Paris and M. Duhamet, a mixture of amino acids and vitamins that can replace the coconut milk. This is true for crown-gall tissue of *Scorzonera* but not true for normal carrot tissue and shows again the difficulty of finding the exact meaning of the activity of coconut milk.

Finally, I would like to point out that there is no relation between the nutrient requirements of plant tissue cultures and of animal tissue cultures. In plant tissue cultures auxin seems to be very important, while in animal tissue culture auxin has no effect. But there is one point of similarity according to the discoveries of Skoog and his collaborators. We know that certain substances extracted from nucleic acid can act on plant tissues, but here is a curious fact: In this country, Klein, and, in Belgium, Manil have shown that nucleic acid promotes only an abnormal type of growth. It has been shown that the properties of the cell can be changed by the use of nucleic acid, in crown gall, in the phenomenon of anergy, and so on. With the nucleic acid, the tissue is no longer normal; it becomes abnormal. Perhaps this can be compared to the facts obtained in the field of animal tissue cultures. I should be glad to know if, in the animal field, nucleic acid does not also promote an abnormal type of growth.

Dr. Ball: I consider the matter of autoclaving the constituents of the culture medium extremely important. Possibly we have gone astray by uniformly autoclaving everything that goes into the medium. For the past few years in my laboratory, we have autoclaved only the agar part of the culture medium. The other constituents, which include the minerals, the sugars, the vitamins, and hormones go through fritted glass. I disagree with Professor Street in his tentative conclusion that such nonautoclaved media will often not support growth. I have grown a wide variety of tissues on such filter-sterilized media and the precision of growth and the duplicability of results is much enhanced. So far as I can discover, in only one case has growth decreased significantly as a result of using unautoclaved culture media.

One other point about the utilization of sugars: Some tissues, particularly some of the crown-gall tissues of *Vinca*, have been grown on 6 different sugars, 3 of them monosaccharides; and, so far as I can determine, the growth rate is about the same for all of them. They were not autoclaved.

Dr. Nitsch: I am in complete agreement with Dr. Ball in recognizing the importance of better techniques. With Jerusalem artichoke tuber tissues, we have observed an effect similar to that reported by Dr. Street. Autoclaving the sugars greatly affected the response of these tissues. Sucrose, for example, seemed to be better than either glucose or fructose when not autoclaved; after autoclaving, however, glucose became far better than sucrose or fructose. Also, if glucose was added to a medium already containing sucrose and was autoclaved, it gave added growth-promoting properties to the medium.

Dr. Thimann: I would like to make 3 specific comments on the remarks made by Dr. Street. First, in regard to the glucose-sucrose situation: Many years ago, Dormer and Street suggested, as Dr. Street mentioned, that the superiority of sucrose is due to some sort of a phosphorolysis phenomenon occurring at the membrane, and the actual entering material is the resulting hexose phosphate. We examined that in a number of ways—not with roots but with coleoptile tissue. I must say that we have found no support for this idea whatsoever. If the entering material were the hexose phosphate, one might expect that, in a system limited by carbohydrate, hexose phosphates would promote growth as effectively as sucrose. The coleoptile provides excellent material for this because its growth in the presence of adequate

auxin is very strongly limited by carbohydrate supply and, in particular, is enormously enhanced by sucrose. However, no hexose phosphate that we have tried has any effect whatever. This is shown both in failure to promote growth and by phosphate analyses, which show no uptake. It may be mentioned that in such systems insulin has no effect, either in promoting or inhibiting the uptake of carbohydrate, and the peculiar inhibition exerted by phloridzin, which Dr. Street mentioned, is apparently not exerted on the uptake of carbohydrate. This can be shown by the simple experiment of treating the material with sucrose for several hours and then removing the source of sucrose, after which growth will take place quite well. If phloridzin is added after the sucrose has been removed, the inhibition exerted on growth by phloridzin is exactly the same as if the phloridzin had been administered in the presence of the sucrose. Therefore phloridzin does not exert its growth inhibition through acting upon the sucrose uptake. I can only say that if this phosphorolysis occurs, it does not occur in the cell membrane. It may occur in the interior of the cell but there is no evidence that it is an entry phenomenon.

The second point is in regard to the chelation phenomenon. Just recently it was reported by Dr. Heath, in England, that chelating agents do promote growth in this same coleoptile tissue, and the suggestion was made that the whole action of auxin on growth rested on the chelating ability of such a substance as indoleacetic acid. Dr. Heath commented on the peculiar structure of indole, which has a nitrogen atom adjacent to an aromatic ring, as does, for example, hydroxyquinoline. It seems fairly clear that this suggestion cannot hold very much water. It is certainly not the mode of action of auxin. We have confirmed that there is a small effect of chelating agents, such as pure sequestrene, on growth. But this small effect, far from being of the same type as that of auxin, is actually shown best in the presence of suboptimum auxin concentrations. In this connection, I should like to ask Dr. Street whether one should consider not only the requirement for metal in these cultures but also the effect of a metal on the growth. One is prompted to say this after studying the very dramatic effect of cobalt on the growth of coleoptile sections. It is well known that cobalt is not required for the growth of whole plants and, indeed, it has not been shown to be required either for the growth of the oat plant or for the growth of isolated sections. Nevertheless, it does increase the growth by 50 percent. This increase cannot be duplicated by any other metal, and it is not, I think, a secondary effect. It depends on cobalt itself and the optimum concentration is low. We have limited ideas of how it occurs.

Third, Dr. Street mentioned in closing that it would be well to examine some of these tissue cultures by more recent methods to see whether, in fact, they do synthesize auxins. We have recently made such an examination using a culture of corn endosperm, which requires yeast extract for its growth, and we naturally suspected that the yeast extract might be required in part for its auxin content. The culture, after a certain amount of growth, was found to contain 50 times as much indoleacetic acid as was present in the original yeast medium (even assuming that it absorbed all that was present) so that it had synthesized large quantities. Furthermore, indoleacetic acid was the only indole derivative found in any quantity and the only auxin. Therefore this does answer the question whether a tissue actually synthesizes indoleacetic acid.

Dr. Street: In answer to Dr. Thimann I would say that I am also skeptical about the Dormer-Street hypothesis because of our later experiments referred to in my paper. The monosaccharides apparently are not inaccessible to the oxidative mechanisms of the cell, because, if you starve excised roots of sugar and then supply the monosaccharides, there is built up quickly a respiratory level similar to that occurring in the presence of sucrose. There may still be an impedance of monosaccharide uptake for we can postulate that this oxidative degradation of the monosaccharides is something that is occurring at the cell surface. Despite Dr. Thimann's comments, one cannot necessarily eliminate the idea that there may be an enzymatic or even a phosphorylative mechanism of uptake. I know, of course, that the commonly available sugar phosphates are incapable of replacing sucrose; in fact, in our experiments,

sugar phosphates were highly toxic. This is a difficult point. It may be a question of exactly where the sugar phosphate is formed by the cells. It is, for instance, a common observation that a whole range of amino acids, which are naturally present in cells, inhibit growth even at concentrations that are certainly lower than the concentrations of the amino acids in the vacuole. Therefore the fact that a substance added externally to a cell is a growth inhibitor does not necessarily mean that the substance is not a metabolite, even an important metabolite, but it may naturally arise at a point that is not actually right at the external surface, and, at the cell surface it may be toxic. Of course, we have always used the orthophosphates of the sugars. Probably you know the suggestion made by Rothstein, I believe, that orthophosphates are not necessarily the only metabolically important phosphates. We have not been able to try glucose-*meta*-phosphates, and it may be that the phosphate species, which are operative and would readily penetrate the root cells, have not been tested. We have therefore to bear in mind that the toxicity of orthophosphates added externally does not necessarily preclude the possibility that phosphates are formed as part of the mechanism of uptake.

With regard to phloridzin, there seems to be a genuine disagreement between the results of our 2 laboratories. The thing that struck us, which we recorded in our paper and which may have led us to arrive at an incorrect conclusion, was that we found a very marked difference in the sensitivity of the seedling root to phloridzin as compared to the excised root. We were able to find a concentration of phloridzin, which was without inhibitory effect on the growth of the seedling root, receiving its organic nutrients and its sugar from the food reserve of the seed down through the phloem, but which strongly inhibited the growth of the excised root receiving exogenous sucrose. We also found that the effect of phloridzin was very quickly reversible; it was so reversible, in fact, that we grew roots first in phloridzin and then in sugar and then in phloridzin and then in sugar and so on. When we harvested them, you could see exactly which part had developed in the presence and the absence of phloridzin. When excised roots are carbohydrate deficient, they are thin and translucent; when they receive an adequate carbohydrate supply, they are thicker and white in appearance. Along the roots described above, you could see that in the absence of phloridzin the growth, degree of vacuolization, and development of air spaces were characteristic of when adequate carbohydrate is available and alternating with these regions were those developed in phloridzin and showing all the evidences of carbohydrate deficiency. We also found good evidence of a competitive inhibition between sucrose and phloridzin; we could counteract phloridzin inhibition by enhancing the external sucrose concentration.

The other observation that requires explanation is that there seems to be a very strict quantitative relationship between the appearance in the external medium of monosaccharides and the uptake of sucrose. And this is something that does not occur independent of the sucrose uptake. There just is not, as far as we could see, any continuous breakdown of sucrose irrespective of absorption. At all levels of sugar depletion, we were able to find a similar relationship between amount of sugar which had been absorbed and the amount of monosaccharide which accumulated in the external medium. This has to be explained, and, while the exact nature of the mechanism is obscure, I still think that the evidence would point to an enzymatic mechanism of absorption.

I believe I have been misunderstood somewhat about the examination of tissues for their hormone content. What seems to be important, and I assume Professor Gautheret will agree with me, is that he can "anergize" his tissues not only with indoleacetic acid but also with a number of other auxins. All the physiological evidence suggests that the tissue which has become anergized has developed an ability to synthesize auxin. Now what is the exact nature of the auxin that is synthesized? Will naphthaleneacetic acid, or 2,4-dichlorophenoxyacetic acid (2,4-D), or other compounds, which are able to cause anergization, create in the cells an ability to synthesize IAA? I know that the anergized tissues have been shown to have a higher hormone content as judged by the hormone assay methods. My suggestion

was that we should look critically at the whole hormone spectrum in the anergized tissue as against the spectrum in the normal tissue. We have recently been examining excised roots for their hormone content and they contain very, very little indoleacetic acid; the presence of the nitrile is also doubtful. The fact is, nearly all the growth-promoting activity as assessed by the standard hormone assay methods is not taken out into the ether but is left in the aqueous fraction. At the moment we have anything but a comprehensive picture of the hormone spectrum in these cultured tissues and it is the development of such a picture that I was calling for in relation to an interpretation of the phenomenon of anergization.

I agree entirely, of course, with Dr. Thimann that when we think of the effects of microelements on growth, we should not think of them only as nutrients.

I would also like to comment on Dr. Ball's remarks because they raise an important question of technique. I do not know the exact details of Dr. Ball's experiments, but I am not at all surprised at his saying that an entirely aseptically prepared medium containing sucrose will promote growth. There are certain points that I should emphasize about our technique. In general, we prepared aseptically only the sugar, and we autoclaved the inorganic constituents and then mixed the 2 fractions. When we prepared the whole medium aseptically (as described by Dr. Ball), then iron availability was not impaired. It is the autoclaving of the whole medium minus sucrose that renders the iron unavailable, and the aseptically prepared sucrose does not contain the necessary organic acids to chelate and make the iron available. During the mixing of the constituents of White's medium, it is clear that a chemical interaction occurs between the ferric sulfate and the 'vitamin fraction.' This combination is apparently destroyed during autoclaving in the presence of the inorganic salts but functions as an iron source in medium not heated at any stage during its preparation.

Dr. Woolley: I would like to comment on the analogy, here, with work done in bacterial nutrition. For many years it has been known that some bacteria are caused to grow by autoclaved sugar. This was first studied in the 1930's by Orla Jensen, who ascribed his particular effect to pyruvic acid formed from the glucose. Recently Dr. Woods, of Oxford, has examined this question with respect to *Escherichia coli*. The active ingredient there, from the autoclaved glucose, is glycolaldehyde. I wonder whether such experiences might not induce one to think that more is involved than just organic acids. I feel that it has been interesting to see how both the plant physiologist and the microbiologist have come up against the biological activities of heated glucose.

Dr. Skoog: I think that as far as plants are concerned, the tissue-culture method is singularly unsuitable for the study of mineral nutrition and that its unsatisfactory nature is clearly demonstrated by the record of recent achievements. In the first place, as to quantitative work, the amount of growth obtained per unit volume of medium is usually small, and the necessity for organic substrate makes it extremely difficult and impractical if not theoretically impossible to remove traces of unknown elements down to the required threshold levels. In the second place, the uniformity of the tissue, even of callus, is very poor. The nutrient probably is used first in one region and then in a younger one, so that actually one can get no real notion of how much nutrient is used per unit of growing tissue.

By the use of algae cultures and water cultures of higher plants within the last few years, a number of elements have been found to be essential for plant growth: vanadium, molybdenum, chlorine, and, for some algae, cobalt; also progress has been made on the problems of nutrient uptake, accumulation, and translocation in tissues. As compared with this, to my knowledge, nothing has been found by the use of tissue cultures. In the case of some elements, Mo, Cl, Co, requirements are so exceedingly minute that chances are they would never be found in tissue cultures even though they exist.

The mere fact that plant tissues *in vitro* require the same nutrient elements for growth as do intact plants and that some kind of nutrient balance must be maintained in the solutions hardly constitute a significant contribution to the science of mineral nutrition.

Turning to the effects of cobalt, which Dr. Thimann studied in *Avena* sections, these were revealed by Miller in excised bean-leaf tissues but by no means in tissue cultures. He also showed that the optimum requirement was too high to be accounted for in terms of an essential nutrient function of cobalt and, furthermore, that either nickelous ion, though less effective, or organic substances may be substituted.

Dr. Earle: As one who has worked in the animal tissue-culture field I would like to ask these gentlemen who referred to autoclaved material to clarify their technology just a little. Is the material used in culture media sealed in glass? Entirely sealed? The reason I emphasize the point is this: I am very painfully aware of a couple of disturbing incidents that have come our way. First, on examining 1 of the old autoclaves that we were using, we found that the block-tin lining was gone and we were using an autoclave that had a beautifully corroded brass lining. A second point, which I think is worthy of some emphasis, is: What is the source of the steam that is used in your autoclave? To those of you who have not looked into this point I may say that if your steam is coming from a central heating plant, your heating engineers are confronted with the problem of keeping the pipes of their lines from corroding. On a recent checkup of our own organization, I have found that there were about 5 components added to the waters of the boilers. One of these, cyclic hexylamine, is added because it is a volatile material; it is carried over in the steam, liberates ammonia, and maintains the steam at a *p*H of about 11.0 to 11.5. Unless you have your material sealed in glass, you have the possibility of either a *p*H effect on the solutions or the chemical action of decomposition products of some of these cute little materials that the heating engineers have to add to maintain your steam system operating. Will some of the plant workers please clarify how they autoclave their materials?

Dr. White: Without attempting to clarify that, may I ask: Do you always autoclave material sealed in glass?

Dr. Earle: We autoclave no solutions used in culture media.

Dr. Lwoff: I was very interested in Dr. Steward's statement that a "root is a root." Then we heard Dr. Gautheret's talk and I thought that one could extrapolate and state that a callus is a callus. Now we learn that a callus may produce roots. Is this correct?

Dr. White: Sometimes.

Dr. Lwoff: And the nutritional requirements of roots arising from a callus are very different from the nutritional requirements of the cells of the callus itself?

Dr. White: And that is reversible.

Dr. Lwoff: Is something known about the requirements of the individual clones that can be grown from callus or from roots?

Dr. White: May I make one comment. There seems to me one thing that has emerged from this discussion and has only been touched upon without being emphasized. In these plant materials, we are almost invariably dealing not with the behavior of individual cells or cell layers but with relatively complex masses in which the mass effects are an important factor. They are much more comparable to Dr. Leighton's cultures in sponge. We are not dealing, in general, at the cellular levels, which you, Dr. Lwoff, are talking about. We do not have, so far as I know in any case, clones in the sense of clones from single cells nor are we dealing subsequently with the behavior of masses that can be considered as single-cell masses as in a single-cell layer. We are dealing with mass phenomena almost invariably. From the nutritional point of view, I think that is a great weakness of the plant side of the work, which we need to overcome.

Nutrition and Metabolism of Animal Tissue Cultures[1, 2]

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Listening to Dr. Street and the panel members who followed him, I was struck by the similarity between the problems that confront those who are studying the nutrition and metabolism of plant cells, and the problems of those who are studying the nutrition and metabolism of animal cells. I began to feel that many of the things I had planned to say about animal tissue cultures hardly needed to be said, because the same problems had arisen, and been discussed. For example, Dr. Street spoke of the relationship of the nutrition of cells of the plant to the nutrition of the plant as a whole, and we shall consider what relationship the nutrition of animal cells may have to the nutrition and metabolism of the animal as a whole. I found myself agreeing strongly with Dr. Street's statement that what we put into a nutrient medium may not be the same as what actually reaches the cells. This is a distinction that we also shall explore.

It will be convenient, and not too great an oversimplification, to define "nutrition" as the external needs of the cells and "metabolism" as what the cell does with the nutrients supplied to it. There is clear biochemical and physiological evidence of a division of labor among the cells of the organism. Both the nutritional requirements and the metabolism of particular cell types are certainly different. We cannot treat a particular cell or population of similar cells, isolated from the animal, as if it were the whole animal. Precise details of the differences in nutritional needs between different cells *in vitro* are still scarce. In discussing these needs, we ought to be agreed upon what are "nutritional needs." The question is, "needs for what?" It is most important to keep clearly in mind that the needs of a particular cell population, to enable it to survive indefinitely in a state of dynamic equilibrium—cell loss being compensated by cell division—are not the same as the needs of a similar population of cells for active proliferation or of a third similar population for engaging in a specific function, *e.g.*, the production of a characteristic external secretion. A cell population that is proliferating has to be supplied with materials from which new cells can be built up. Cells that are differentiating or

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fulfilling some specialized function have to be supplied with nutrients which they can metabolize to produce the hormone, pigment, or other characteristic product of the particular cell type.

It may be argued, and as recently as 10 years ago would certainly have been commonly contended, that the difference between conditions for *survival* and conditions for *proliferative activity* resides in the absence or presence of one or a few components with the particular function of stimulating cell proliferation. For at least 20 years, from about 1920, much effort was put into the search for these "growth-promoting substances." I notice that such terms are still used freely by the plant tissue culturists, and I wonder whether the concept of the "growth-promoting factor" should not be carefully re-examined to see if it still holds a useful meaning.

If one has a set of conditions that will permit a population of cells to proliferate and one removes something, or alters the *p*H, or in any one of a number of ways changes these conditions, the nutrient can be made ineffective for growth. To take an extreme example, by omitting sodium chloride from a medium otherwise sufficient for growth, the medium becomes inadequate not merely for growth but for supporting life. But unless terms are more carefully defined than is usual, we might be led to regard sodium chloride as a growth-promoting factor—for, in its presence, the cells will grow. Many other substances can be omitted and permit more or less prolonged survival without growth. Any one of these might be included in the definition of a growth-promoting factor. Because of the multiplicity of substances that fall into this category, one should not speak of any substance as *a*, much less as *the*, growth-promoting substance, except in relation to the over-all pattern of the metabolic environment. I have tried before (*1*, *2*) to throw light on the obscurity enveloping the "growth-promoting factor" idea, by quoting a statement made by the microbiologist B. C. J. G. Knight (1945). This clarifies the relationships between cells and the nutrients supplied to them so usefully that, at the risk of laboring the point, I want again to draw attention to it. In his words, ". . . a given substance, required as a component of one of the essential metabolic processes, might appear in three different roles . . . (1) as an 'essential nutrient,' when its rate of synthesis by the cell was so slow as to be insignificant; (2) as a growth stimulant, when its rate of synthesis was somewhat faster but still slow enough to be a limiting factor; or (3) as a substance not required at all for nutrition, because the cell could synthesize it so fast that it was not a limiting factor in growth. It is the metabolic process which is the essential thing."

This seems to me to be a statement of the utmost importance, not only in microbiology but also in studying metazoan cell nutrition and metabolism. If it is not heeded, we can expect to find in the literature statements about substances categorically described as essential nutrients or as growth-promoting substances, which are meaningless unless they are seen in relation to the rest of the total activity and environment of the cell population. These terms refer to no absolute properties of particular

chemical substances. It may very well be that there is not just one set of conditions under which one could obtain, *e.g.*, maximal growth of a culture of cells. There are undoubtedly several ways of achieving this. The cells themselves can and do alter the external conditions—much more effectively than is sometimes realized. And, following from this, is one to name a particular substance "A" an "essential nutrient" if the cells transform it into a substance "B," which could equally well be utilized if supplied directly? The important question here is not whether "A" or "B" is the "essential nutrient," but whether one should allow the cells to exercise their capacities to form "B" from "A." So, although the nutrients and their interrelationships are important, "It is the metabolic process which is the essential thing."

The synthetic capacities of the most-studied metazoan cells are more limited than those of most microorganisms and plant cells about which we have information. For example, animal cells require more than a single, simple nitrogen source, *e.g.*, glycine, ammonium salt, or nitrate, which may suffice for some bacteria or higher plant cells. But this should not be taken to imply that animal cells are not capable of a multitude of synthetic steps. Empirically, this raises the question for those who are devising nutrients for these cells: Are we to provide nutrient substances as nearly as possible "ready-made" for living and growing cells? Or are we to allow the cells to exercise, to the limit of their ability, their inherent capacities to synthesize complex from simple metabolites? For example, if a cell can synthesize cocarboxylase from thiamin and phosphate, should we supply these components or the coenzyme preformed? Is thiamin the "essential nutrient" or is cocarboxylase? In many cases one might be just as effective as the other. It would then really not matter, empirically, which was supplied. But by supplying the simpler substances we might be able to learn more about the synthetic abilities of particular types of cells. There is no absolute right or wrong answer to this kind of question. Individual answers may be the result of attitude, preference, or prejudice. But it is important to realize that many nutrients are "essential," "useful," or "nonessential" only in relation to the composition of the rest of the nutritional environment. My own preference, at the present stage of development of this field, is to keep nutrient substances as simple as possible and to let the cells perform those synthetic steps of which they are capable. By this means, I feel, we are likely soonest to reach an understanding of the metabolic capacities of the cells we study. But, the sufficient nutrient medium which contains the "simplest" (least complex) components may not necessarily be the "simplest" in terms of the smallest number of known components. Lest my preference may appear as a prejudice, I should add that the opposite point of view, namely, that we may also learn much about the nutritional needs of cells by giving them complex substances which will permit cellular function or growth, is perfectly valid. This is similar to Dr. Steward's approach to the nutrition of plant cells. We can learn from both methods of attack.

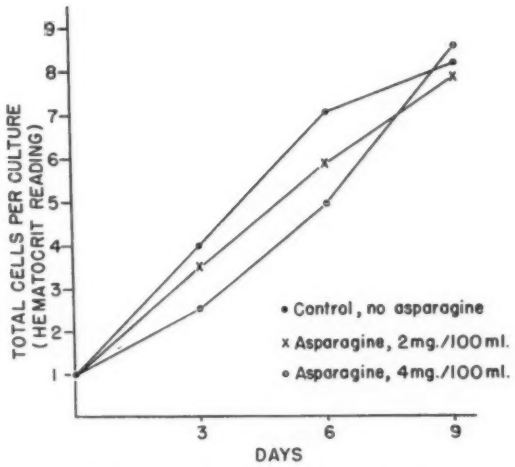
Since absolute requirements for particular substances have no validity unless clearly related to the over-all nutritional and metabolic background against which they are found, it becomes evident that any particular medium that may be devised for any particular cell type may be largely a reflection of the preferences of its author. Fortunately, cells are remarkably adaptable to widely varied environments. The size of a cell population, and therefore its total metabolic activity, is very important in relation to the availability of nutrients in a given medium. This question of cell : nutrient ratio in itself warrants a 30-minute discussion. It is common experience that a nutrient medium which will support the growth of an inoculum of 200,000 cells per ml. may (for different reasons) be insufficient both for 200 cells per ml. and 2×10^6 cells per ml.

For cells *in vivo*, the *immediate* source of nutrients is the interstitial fluid or the circulating blood. The *ultimate* sources are, in part, unmodified constituents of the food; but, for the most part, they are food components metabolized and changed by the cells of other organs and tissues. Thus cells in all parts of the organism, with different metabolic capacities and different nutritional needs, are interdependent and receive their nutriment in the form of a complex biological fluid that is adequate for all but not necessarily optimal for any. From the viewpoint of animal physiology, it is a commonplace to look upon the blood stream as the "universal provider" for the somatic cells. An extension of this thinking to the physiology of explanted cells has given rise to the assumption that blood plasma can provide an optimal diet for tissue cultures. It is a misfortune that the study of cell nutrition has been so circumscribed by this concept which has led to the idea that the optimum environment for the life and growth of cells is the one that, with the greatest ingenuity, imitates the plasma of the blood. It did seem, in the early days, as if this biological fluid, suitably diluted, was rather innocuous to cells. Chicken macrophages, for example, can proliferate in it and retain a "normal" appearance for months or years. But the blood stream is the "sink" as well as the "source" for a wide array of cell products and metabolites. For many cells *in vitro*, plasma and similar biological fluids are not sufficient even for survival, and for most cells they are inadequate for growth. This has long been common knowledge, but the rigid patterns of thought persist, and efforts to devise chemically defined media are often still based on the idea that its constituents, even though fewer in number, ought to be present in the concentrations in which they are found in plasma. This is the more absurd since the moment the cells in tissue culture are brought into contact, either with genuine plasma or with one of our chemically imitative substitutes, the composition of the medium begins to change. Metabolism, in short, comes in. Some medium components are liable to be rapidly used up, and a single deficiency may render a medium wholly ineffective. *In vivo*, the plasma is constantly renewed and replenished, so that on balance its constitution remains fairly stable. Unless we perfuse our tissue-culture cells with serum or the serum model at a rate com-

parable to that at which tissue fluid reaches the cells in the body, the imitation is incomplete. What we usually do is supply the cells with a limited volume of medium and replace this with fresh medium after 1, 2, 3, or more days. Some constituents, usually we do not know which, are by then exhausted. We are now beginning to realize that, well within toxic limits, some nutrients can be supplied at concentrations far above the so-called "physiological" concentrations found in plasma. Plasma and serum have served their day as crude approximations in exploring the cells' needs. From now on, nutrients will be made to fit particular cells; and we shall allow the cells to do a great number of things for themselves with rather simple mixtures that will probably diverge greatly in composition from biological fluids.

In trying to do better than imitate biological fluids, how should we supply nutrients to our cultures? Perhaps an ideal arrangement might be to adjust the rate of supply to the rate at which substances are taken up or used by the cells. For a growing culture, this would mean continuously increasing the concentration of the nutrients as the size of the cell population increases. We do not do this, nor, probably, would we be justified in trying to devise a means of doing it with our present incomplete knowledge of cellular nutrient requirements. With our present methods of adding a fixed volume of nutrient medium to a culture and renewing the nutrient at intervals, we run into interesting problems in trying to achieve so-called "optimum" concentrations of single substances. For example, we have made some observations on the effect of asparagine, at 2 concentrations, on NCTC clone 929 of strain L cells (in the presence of salts, glucose, 6 B vitamins, hypoxanthine, ascorbic acid, glutathione, glutamine, 16 amino acids, and a mixture of peptides). The growth curves over a period of 9 days, measured in terms of the total volume of cells per culture flask and estimated in a hematocrit (*3*), are shown in text-figure 1. At low cell populations per unit volume of medium, the addition of this substance depresses cell growth. Lower concentrations have a less inhibitory effect during the early part of the culture growth, *i.e.*, while the cell : nutrient ratio is low. By 9 days, however, cultures exposed to higher concentrations of asparagine have grown more than controls or than cultures in a lower concentration of the added substance. If one were thinking in terms of "growth-promoting substances," one might conclude, at 6 days, that asparagine was inhibitory but at 9 days that it was stimulatory. Exactly the same pattern of growth curves can be demonstrated with different concentrations of other substances, *e.g.*, with a mixture of amino acids or with magnesium ions.

In conclusion, there are probably more ways than we have suspected for achieving media for survival or growth, depending on how much or how little the cells themselves are called upon to modify the nutrients. And we can improve upon plasma, and other biological fluids, in different ways for different cells, by devising media to bring out the latent capacities of the cells. We look forward to being able to manipulate the nutrition of cells to elucidate a range of metabolic functions. A proper combi-



TEXT-FIGURE 1.—Effect of asparagine on total cell population of NCTC clone 929 of strain L cells.

nation of such functions—and there may be many such combinations—will result in growth. Let us forget about "growth-promoting substances" and concentrate upon metabolic processes.

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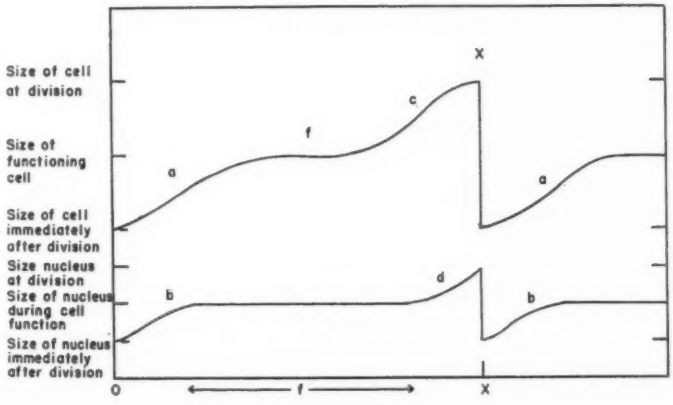
DISCUSSION

Dr. Willmer: Although I agree with Dr. Waymouth in nine tenths of what she has said, I would like to make two comments. First, I would like to discuss with her the question of growth-promoting substances. I think, in a sense, she has made the system too simple by eliminating them altogether. I like her classification that when we are culturing tissues we may have three ends in view. We may be trying to keep the cells alive and differentiated morphologically; or we may be trying to keep them alive and functioning and producing their particular metabolites; or, third, we may be studying cells with the idea of making them grow. Perhaps the distinction that I want to make can be illustrated by the liver. In the body the liver is an extremely active metabolic organ and it undoubtedly can synthesize a great number of substances, but, in the adult body at least, it is not a growing system. If, however, you wound or remove part of the liver, it then becomes a growing system. This may, of course, be the result of removing some inhibitory process, but, it is equally possible that it is the result of channeling the metabolism into some other direction for which different metabolites may be required. For example, if we consider the way in which a cell grows there are two aspects that we ought to keep in mind. Let us say a cell has divided at point 0 (text-fig. 1). The cytoplasm might grow like "a" and the nucleus like "b," and the cell might continue to function for a time without dividing again—"f." Alternatively, the cytoplasm might grow like "c," while the nucleus grows more like "d." At "x" the cell divides and the process repeats itself. It seems to be fairly clear that there are two distinct activities in such a situation—the primarily cytoplasmic function and the production of nuclear material. In tissue cultures it is perfectly possible to keep cells alive and maintaining their weight for a considerable time without any growth by cell division at all. When you add certain tissue extracts, like embryo juice, you alter the whole system and throw the cells into what one might call the growth phase. The embryo juice seems to affect nuclear synthesis and may alter cells toward nuclear construction. This is probably different from the kind of cytoplasmic synthesis that would lead to the production of things like enzymes and secretions in the cytoplasm. In fact, I think we ought to keep separate the metabolites that are necessary for the functioning of a cell, *i.e.*, the complete functioning of the



TEXT-FIGURE 1.

cell, including its capacity to build all the things it normally does, and those substances that can change the cell from a functioning into a growing cell. I wouldn't like to rule out the possibility that there may be specific substances necessary for this. In the case of the liver, I may have chosen a bad example, because there it may be a removal of some inhibitory process that causes growth. But in cell activity there are certainly two main phases that I personally would like to keep separate, while maintaining a little cubbyhole for growth-promoting substances—at any rate as a possibility.

The second point I would like to make with regard to the metabolism or nutrition of cells *in vitro* is one that has come very much to the fore recently with the discovery that it is now possible to keep single cells alive and growing. I think there are two things that are extremely important to bear in mind. One is: What is the real nature of the cell that we have isolated? In the body many cells are very closely dependent on each other, and when you isolate a cell from its particular situation you thereby alter its whole activity. For example, most epithelial cells only function as epithelial cells proper when they lie on a basement membrane that is formed under the influenced of fibroblasts or connective tissues underneath. When such an epithelial cell is isolated it is deprived of its morphological shape and it is very likely to be completely disoriented and to start to work quite differently. When clones of cells are produced the investigator is immediately working with cells in a completely different environment from that in the body, and may be measuring something rather different from anything one might measure in the normal situation in the body. Second—and this has become very obvious to me, since I have been fortunate enough to travel over this country and see a large number of tissue-culture strains grown by different people in different places—cells, when isolated in these clones, almost certainly change their character and become very different from what they were in the body. When making these clones, it is thus very important to bear in mind what type of cell is being grown and how much it has become modified. For example, I have seen cultures of liver cells growing very actively and perfectly healthily, but as quite isolated cells unlike normal epithelial cells. I have also seen cultures of liver cells of the same age in the same kind of medium growing as continuous sheets of epithelium, and there was no doubt that we were at least dealing with true epithelial cells, if not with immediately recognizable liver cells. The results of metabolic studies on the first are likely to be different from those on the second. Which result is "right," if either, of course, remains for the future.

Dr. Parker: I agree with Dr. Waymouth that we should re-examine the problem of the food requirements of explanted cells. And we must not forget that explanted cells can seldom be identified with their ancestors in the body. It is even possible that everything that happens to them, after explantation, affects in some way their response to a given environment. In our laboratory, we worked up a particular medium (CMRL-858) with a particular line of Earle's L strain cells and could obtain 9- and 10-fold increases in the population in 7 days (*1*). This line eventually became contaminated with bacteria. We treated it severely with antibiotics and obtained a clean line, but it never behaved the same again. Nor have we obtained the same high response with other L strain lines. Swim and Parker, of Western Reserve, have recently made experiments that show how important every detail of the life history of a strain can be in nutrition work. If Dr. Swim, who is here, would describe some of their findings, I am sure you would conclude that a laboratory developing a new medium should always supply the matching strain of cells, along with the formula, to interested colleagues.

Dr. Waymouth has suggested, quite rightly, that a particular medium devised for a particular cell is as much a reflection of the author's requirements as of the nutritional needs of the cell. Biochemists making turnover experiments and microbiologists studying host-cell relationships are usually satisfied with a medium that will provide a reasonably high rate of multiplication over a relatively short period. Others are content if their cultures may be kept in a relatively stable state of low activity for longer periods. But a particular medium is also a reflection of every detail of a

laboratory's manipulations. Thus, *e.g.*, when conditions are optimal in our laboratory, we can obtain the sort of useful increases I have mentioned in stationary cultures prepared with synthetic media. But when the same cells in the same media are kept in suspension by rotation at 50 to 60 r.p.m. (*2*), there is no appreciable increase in the population. When the same synthetic media are fortified with dialyzed blood serum, the cell population in these agitated cultures increases rapidly and logarithmically. It is probable that the cells in the stationary cultures and in the agitated cultures are in different metabolic states.

Still other happenings affect the metabolism of explanted cells and influence their response to a given medium. I refer to cell alterations or transformations. Some of these changes affect both the form and function of the cells; others are metabolic changes unaccompanied by easily discernible structural changes. Though changes of this kind had been noted earlier, I first encountered them 25 years ago in experiments (*3*) in which chick-embryo cells were cultivated for extended periods in media lacking embryo extract. And when Carrel's old strain of chick fibroblasts, then in its 19th year, was cultivated without extract, it too produced altered cells, which suggested that it was not just a matter of sorting out a mixed population. The Carrel strain had recovered from so many bad periods over the years that it certainly wasn't a mixed population in the usual sense. The altered cells that are best known, of course, are Earle's L strain cells from the mouse, reported in 1943 (*4*). Since then, altered cell strains have been derived from a wide variety of tissues. In our laboratory, cell alterations have been observed over the past several years with almost frightening frequency (*5*). Thanks largely to the efforts of my colleague Dr. Ernest McCulloch, and a former guest, Dr. L. N. Castor, of Philadelphia, we now know a bit more about these changes.

The altered cells are characterized mainly by the fact that they multiply with great rapidity to yield uniform populations of free-living cells that can be centrifuged, washed, and subcultured indefinitely (*6*). Sometimes the changes occur in old cultures in which cell multiplication has ceased; sometimes they occur in healthy cultures. With human bone marrow, they may take 3 to 6 months to occur, provided the cells can be kept alive that long. Monkey kidney epithelium usually requires 2 to 3 months; peripheral-blood cultures, 2 to 8 weeks. Also, Castor found that the time required for the alterations to occur was roughly the same for cultures left in their original containers as for cultures kept in a more active state by subculturing. During recent years, most of our cell alterations have occurred in synthetic media supplemented with heterologous serum, though they have sometimes occurred in media in which the serum supplement was homologous. They have never occurred in unsupplemented synthetic media, though in 1 such experiment we managed to keep monkey kidney epithelium alive for 6 months. We are concerned, of course, about the possible malignancy of these altered cells, but so far all tests for malignancy have been uninformative, despite the fact that we have obtained altered cells from malignant tissues.

Our most recent cell alterations have occurred in clones derived by single-cell isolation from cultures of unaltered monkey kidney epithelium. The single cells were isolated in capillary drops of medium on a coverslip, under a layer of oil in a petri dish, according to a technique devised by deFonbrune and adapted to animal cell work by Lwoff and collaborators (*7*). In an example chosen for illustration, the clone developed as typical epithelium, and the fluid in the drop was renewed twice a week. On the 13th day, when a sheet of about 400 cells had formed, the cells were detached from the coverslip with dilute trypsin and transferred to a stationary test tube (13 × 100 mm.), where they continued to multiply slowly to form epithelial sheets as before. Then, 2 weeks later, several nests of exceedingly active altered cells appeared in the epithelium (fig. 1, plate 39). Cells from one of these nests were then transferred to a larger container, where they continued to multiply with great activity (fig. 2, plate 39). This, then, is an extreme example of metabolic differences between cells of the same clone within the same culture.

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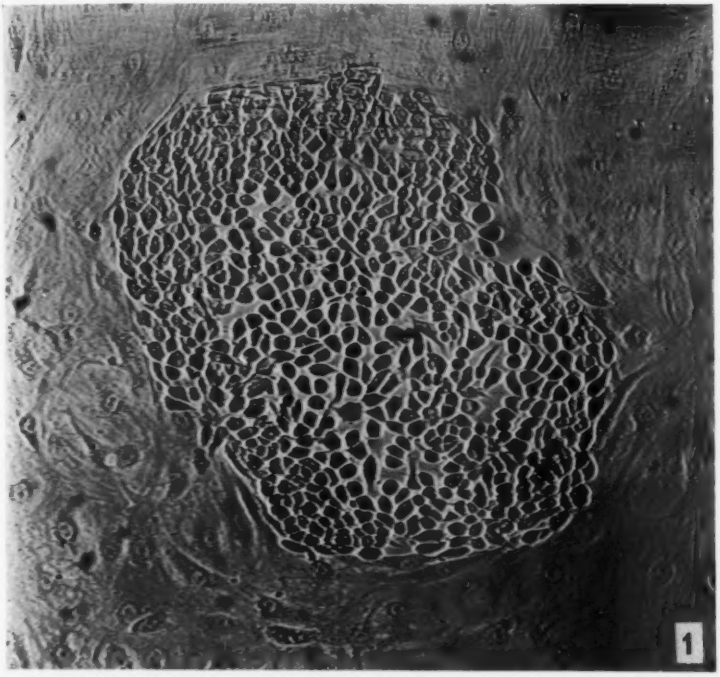
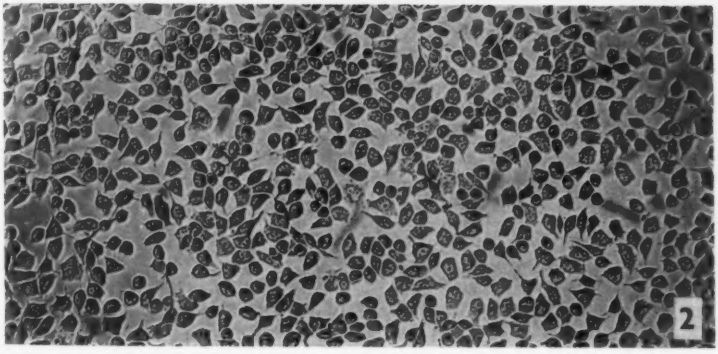
PLATE 39

FIGURE 1.—Nest of altered cells in clone derived by single-cell isolation from culture of monkey kidney epithelium. The clone was left on a coverslip for 13 days, then transferred to a small test tube 16 days before this photograph was made. *Note* unaltered epithelial cells surrounding the nest of altered cells. (Culture 21827) × 120

FIGURE 2.—Six-day-old flask culture (21936-3) derived from 1 of several nests of altered cells that developed in clone shown in figure 1. × 120





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Dr. Harris: Dr. Waymouth and the other speakers have given us some useful perspectives here and have presented some of the problems that are inherent in our present outlook on questions of cell nutrition and metabolism. I would like to contribute further to this general framework of discussion, for it would seem that the interpretation of the facts we study is certainly more important than specific experimental details. My comments can perhaps be put in the form of two general questions. The first of these is: Can we afford to discount the role of extracellular protein in the studies we make of cellular nutrition and metabolism? Dr. Waymouth has presented some very cogent arguments for using media made of the simplest possible constituents. It certainly has been the goal of some of the very best workers in this field to use protein-free media, or at least a medium containing a minimum of protein consistent with growth or maintenance. The value of these approaches is certainly obvious enough. Yet one wonders whether we do not run the risk to some extent of throwing out the baby with the bath. Can this important factor, extracellular protein, be completely eliminated? I think we have to remember that the natural environment *in vivo*, as some of the other speakers have suggested, is one in which the cells are immersed in protein-rich media. One can't assume that intra- and extracellular proteins are functionally separate or even that these cells, if taken out and put in tissue culture, are necessarily able to maintain the integrity of their intracellular proteins against loss through altered permeability. We have been particularly impressed with the latter possibility in our recent work with chick cells freshly isolated from the embryo and grown as a monolayer on glass. These cells may show conspicuous signs of loss or extrusion of protein and, coupled with this, alterations in the population, giant cells, many different variants, and, eventually, a decline in growth rate in serial transfer to an eventual loss of the strain. These alterations are most conspicuous in media containing heterologous sources of serum protein. They are less evident where we use homologous serum. If we supplement homologous serum with a protein fraction obtained from adult chicken spleen we can maintain these populations in an extremely uniform condition, perhaps indefinitely, certainly through very long periods of transfers. We have here a stabilizing factor, one might say, which is very interesting to compare with the points Dr. Parker has just made on cell alterations. One wonders in this connection whether, perhaps in a crude sense, we are dealing with an analogy to a mass law effect as far as the protein is concerned; whether, in the case of freshly isolated strains that do not survive indefinitely as established strains, we have the progressive leakage of proteins, and leaching out of essential internal components resulting eventually in the loss of the strain. Or, conversely, whether established strains represent variants for which the loss of these internal constituents is no longer limiting. There are also indications of a much more dynamic role of protein for the growth and metabolism occurring within the cells. I refer to the experiments *in vivo* with tracers, which have indicated a dynamic equilibrium between plasma protein and the proteins of the cell. There is a very conspicuous uptake of protein from without in certain instances, especially perhaps in the case of tumors. These experiments *in vivo* have their counterparts in the very significant work of Winnick with chick fibroblasts and that of Westfall and his collaborators with HeLa cells, in which a "balance-sheet" approach has been used. One could thus estimate the proportion of intracellular protein derived from the amino acid and peptide fraction, and this, as it turned out, was a minority of the total, perhaps only one third, the greater part apparently being derived directly from the extracellular proteins of the medium rather than from smaller molecules. The mechanisms of this process are quite obscure to us still, and yet these observations are significant in that they cast the extracellular protein in a much more dynamic role than we have been accustomed to assume for it in the past.

The second point on which I would like to comment concerns the area Dr. Parker has just discussed, namely, the alterations in established cell strains. My interest here is in the matter of interpretation of nutritional or metabolic data obtained exclusively with the use of established cell strains. As Dr. Parker has suggested, and as is common knowledge, these strains vary conspicuously from their populations of origin, and certainly the earlier experiments of Dr. Sanford are beautiful in indi-

cating that variation can occur in clones obtained from a single cell. What we are saying here is, of course, obvious from early microbiological studies where population dynamics is a most essential part of studies on growth and metabolism, and one has to consider the possibility of mutation, phenotypic adaptation, or other population changes. We are only now achieving tools in tissue culture that will make a similar precision of population analyses possible for animal cell systems. The point I wish to make here is simply this: Since established cell strains do differ in certain respects, do vary, one can assume that their metabolic and nutritional patterns are likewise subject to change, whether this be by mutation and selection, phenotypic adaptation, or other phenomena not known so far. If we find that the specific nutritional requirements of a particular cell strain *in vitro* differ from those of the intact organism, we are at a loss to know whether this means that the corresponding cells *in vivo* generally have this same requirement—let us say a requirement for the amino acid, tyrosine, as the L cells show *in vitro*—or whether this has been a nutritional change by mutation or other alteration after separation from the body. Perhaps greater emphasis should be placed on the use of freshly isolated cells. In standardized suspensions, as for example with chick myoblasts in our laboratory, these permit quantitative studies and form a useful check on the problem of variation in established strains.

In conclusion, I would simply emphasize that the experimental designs that are methodologically the cleanest are not necessarily the most meaningful in a biological sense and that perhaps we ought to continue study of nutritional and metabolic problems on a broad scale without any arbitrary restrictions on our horizons.

Dr. Waymouth: I would like to comment on some of the remarks made by the panel members—first Dr. Willmer. I don't know whether you noticed that I succeeded in talking about the nutrition and metabolism of animal cells for 30 minutes without once mentioning the term "embryo extract." This would hardly have been possible 10 years ago. Since Dr. Willmer has brought it up, I think I should say that I agree that it is perfectly possible, legitimate, and indeed useful, in some circumstances, to make a distinction between nutritional conditions suitable for growth and conditions suitable for maintenance. But I deliberately tried to keep away from the idea that embryo extracts and such things contain specific "growth-promoting substances" in order to emphasize, as I quoted from Knight, that "It is the metabolic process which is the essential thing." The cells have the capacities to use different nutrient combinations that are presented to them. If they are presented with embryo extract, they will go along one pathway; if they are presented with another nutrient solution, they will go another way. I want to stress the diversity of metabolic possibilities more than has been done in the past.

The second point is that questions of concentration gradients at the cell boundary are most important for growth and also for differentiation. I hope someone who is working in this field will comment on this. As Dr. Willmer showed, contact of an epithelial cell with a basement membrane is a case in point. The maintenance of differentiation probably depends on production of specific substances by neighboring cells. Also, in the development of clones of cells, latent capacities in the cells are being elicited. The way a cell is going to develop depends on the kind of environment it is put in.

Dr. Parker's beautiful observations on the variations in cells under artificial conditions again show that the cells have all sorts of capacities that are usually not brought out but that he has elicited by providing appropriate conditions.

Dr. Harris spoke about extracellular protein. I am sure this is important. There is no question that cells, under normal circumstances, are always exposed to a protein-containing environment. I feel less sure that the only, or even the most important, role of protein is that of a nitrogen source. We should bear in mind the possibility that proteins can act as carriers for small molecules, which may be presented to the cells in this way amd thus be more readily used by the cells than in simple solution. I do not disagree with the evidence that protein can be broken down by the cells and utilized for building up new proteins; but I think we should keep in mind that proteins may have other functions as well.

Dr. Melnick: We have been concerned in this section chiefly with cells and the media used for their cultivation. My colleague, Dr. Catherine Rappaport, has recently found that one must consider another factor—the glassware on which these cells are grown. She has developed a protein-free synthetic medium for growing monkey kidney cells; but for growth in this medium, the cells must be seeded in soft glass such as is used in Brockway prescription bottles; if pyrex 7740 is used, it must first be rinsed with NaOH. Her protein-free synthetic medium contains large amounts of calcium—apparently the calcium may replace the protein in attaching the cells to the glass. A monolayer of cells, which develops in about 5 days after seeding, seems to be different physically from the cells that grow on pyrex glass in the presence of protein in that it has been impossible to dislodge the cells from the glass even with trypsin or versene at a concentration many times higher than that which will take cells off in the presence of protein. Even scraping has been unsuccessful because the cells are ruptured in the process. However, the cells have been carried for a few passages, because after the monolayer is formed, the next cell division forces some of the cells free into the supernatant fluid. Then, by seeding these cells into new bottles, she has been able, in some experiments, to carry them for 3 months.

Dr. Chèvremont: Dr. Waymouth mentioned nucleic acid a moment ago. Perhaps I should add a few words partly answering the question of Dr. Gautheret. Ribonucleic acid (RNA) promotes the growth of fibroblasts and myoblasts of chick embryos. A hydrolysis of RNA by ribonuclease (RNase), on the other hand, eliminates the growth of the cells. We must therefore pay attention to the contents of the medium. Some investigators have noted no effect of RNA. In making investigations I noticed that when the medium was too poor it was not enough to add a substance to the medium. The addition of RNA had no effect. But the opposite was true when the medium was so rich that the cells had everything needed—it did not matter if one added something else. With an intermediate composition of the medium, added RNA promotes growth. With chicken cells this is true even when RNA from yeast is added. Deoxyribonucleic acid (DNA) does not act even if it comes from the same species. As far as the second point is concerned, we all know that RNase will hydrolyze RNA in the fixed tissue of sections. But this enzyme is also able to enter the living cell. It inhibits growth. Basophilia of the nucleus and of the cytoplasm disappears and the nucleoli especially are highly modified. Cells remain in prophase and do not continue mitosis even if the same cell is observed for 20 to 30 hours. Many cells are blocked with the aspect of prophase or beginning of prophase. Finally, the cells disintegrate and degenerate. But it is quite possible to cure this inhibition of growth by adding RNA. For example, if you transplant cells 2 or 3 times in contact with RNase and in the following transplantation you put a few cultures in Tyrode's solution, nothing grows. If you add RNA, even from yeast at a concentration of 1 : 10,000, the culture completely recovers its growth.

Dr. Evans: I would like, in a certain sense, to disagree with Dr. Waymouth and Dr. Parker on the point that each medium is some devisor's particular interest and that a bottle of medium should be provided with each strain of cells sent out. They should not lose sight of the fact that, presumably, a medium is devised to do a specific job under specific experimental conditions and so it necessarily has to represent the devisor's requirements and hence his particular interest.

I would like to point out that to aid investigators and to save them time, it would be a good idea to send out with a given strain of cells the medium devised for the strain of cells. Thus, the investigator has an opportunity to test them together, prior to combining the formulation in his own laboratory. With both the cells and the medium, the recipient is saved from the expenditure of time required to go through the procedure of stabilizing the strain in his laboratory to provide a substrain on the chemically defined medium.

All of us who have been in this field for an appreciable length of time know that it is not possible to get every explant from fresh tissue to grow even on a naturally occurring medium. Nor is it possible to get every culture of an established strain to grow as a substrain indefinitely on a chemically defined medium. In the past 6 months,

however, we have had a medium with which we have achieved some success, specifically medium NCTC 109, in growing indefinitely more and more substrains of strain L. It proves, in a certain sense, that we are slaves of our own technology, because it seems to demonstrate that as we improve our technology in handlIng cells on this medium we reap the rewards from such improvements.

Dr. deBruyn: May I comment on Dr. Harris' lecture about the extracellular protein? We are working with a strain of cells that has been derived from a subcutaneous transplantable tumor of the mouse, a lymphosarcoma (MB T 86157). The cells are in suspension, growing in a medium that contains only 25 percent horse serum or human cord serum and 75 percent Tyrode's solution. We count the cells before and after 4 days' or 1 week's growth. The total nitrogen is measured to see whether this corresponds with the number of cells. We also measure the free amino acids in the medium. After 1 week's cultivation there are 2 or 3 times as many cells and the amount of the free amino acids in the medium has also increased. We must assume, therefore, that in this case, with this kind of cell, this medium, and this method, the cells have used up some of the protein from the serum that was given to them.

Dr. Swim: Investigations concerning alterations in the characteristics of cells that occur *in vitro* have centered on morphological changes, and little attention has been directed to variations in the nutrition of cells that may occur during the course of prolonged serial cultivation. In studies on the general nutritional requirements of mammalian cell cultures, we observed that cultures frequently acquired the ability to proliferate in media which initially appeared to be inadequate. These results indicated that such cultures contain a small proportion of cells capable of proliferating under conditions unsuitable for the remainder of the population and that a culture is able to adjust to a new environment as a result of selection of the appropriate nutritional variant. To examine this possibility in more detail, the following experiments were performed. Fibroblasts, strains U12 and L, which are propagated in a medium containing 5 percent chick-embryo extract and 20 percent horse serum, were placed in experimental media lacking 1 of these components. Under these conditions, the cells gradually degenerated until it appeared that most of the cells were involved. After several weeks, however, a few healthy cells were noted in each of the cultures, and their progeny continued to proliferate luxuriantly in the experimental media. On the basis of growth response in several media, it appears that the nutritional requirements of these sublines or variants of L or U12 differ markedly from each other and from the stock cultures from which they were derived. A nutritional variant has also been isolated from a culture of rabbit fibroblasts which can be propagated serially in a medium containing 2 percent dialyzed serum as the only undefined constituent, whereas the parent strain requires whole chick-embryo extract and serum for growth. The results of these experiments indicate quite clearly that the nutrition of a particular strain of cells is, in part, a function of the composition of the medium in which it is propagated. Furthermore, they point up the importance of recognizing the possibility that many permanent strains of cells are laboratory artifacts, insofar as their nutritional characteristics are related to those of the cells originally established *in vitro*, and that the results of nutritional experiments with a strain of cells may differ in several laboratories due to variations in the composition of the media employed to propagate the cells. These are problems which the microbiologist encounters constantly and it would appear expedient to consider the degree to which information available in this area may be applicable to cultures of mammalian cells.

Nutrition and Metabolism in Fields Collateral to Tissue Culture [1,2]

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I have been asked to discuss nutrition and metabolism in collateral fields. Like a number of scientists I have often felt definitely collateral. Thus, I was very sympathetic with collateralism in general. The difficulty started when I tried to figure out what sort of collateral talk could be produced before a collateral audience composed of cell biologists, including also those lateral people whose main interest is to kill cells and who are generally referred to as virologists.

An arbitrary decision had obviously to be taken which was likely not to satisfy anybody, including myself. It was decided to discuss a few data concerning bacteria and especially the functioning of the bacterium as a whole.

The study of metabolism is generally restricted to the chemistry of reactions involved in energy transfer or in the synthesis and breakdown of various substances. Traditionally, scientists interested in metabolism do not study growth and *vice versa*. A gap exists between both fields and few people have ventured to penetrate into this no man's land. Those few, however, have harvested fascinating results and, as a matter of fact, open the way to developmental physiology at the molecular level. This rather new branch reaches, of course, far beyond bacteria and participates in the generality of biology.

My intention is to consider, first, the problem of the induction of enzyme synthesis, then the problem of the inhibition of enzyme synthesis. The conclusion will be reached that regulation of enzyme synthesis might be the result of the competition of inducers and anti-inducers for a common key site. This may be true also for the enzymes which control the concentration of various substances in the bacterium. The existence of these enzymes allows us to understand the nature of bacterial memory, which is bound to a steady state. Analyzing further the interplay of essential metabolites, we will discuss the role of amino acids in the synthesis of nucleic acid. We will then consider a few data concerning incorporation of analogues in its relation with specific key sites. This will bring us finally to the problem of abnormal growth.

[1] Received for publication May 28, 1957.

[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8–12, 1956.

My ambition is to cement these apparently heterogeneous subjects. The leitmotiv of this paper will be the interplay of metabolites and antimetabolites in the balance of synthetic processes as it depends on specific key sites.

Generalized Induction Principle

Induced biosynthesis of enzymes has, for a long time, appeared as an exceptional phenomenon restricted to a few dispensable enzymes. Today induction is known to be involved in essential systems and seems of general and fundamental importance.

The study of induced biosynthesis of enzymes has brought interesting information concerning the control of protein synthesis in general and the problem of cellular balance in which we are interested. The most complete set of data concerns β-galactosidase. A few years ago only substrates of the enzymes were supposed to exert an inducing activity. It has since been shown that such substances as phenyl-β-D-galactoside which are metabolized have no inducing power. Conversely, some substances like melibiose are excellent inducers but are not metabolized as carbon and energy sources (*1*).

TABLE 1.—*β-Galactosidase of* Escherichia coli *ML 30 lactose* +

	Enzymatic activity		Synthesis of the enzyme	
	Substrate	Antisubstrate	Inducer	Anti-inducer *
Methyl-β-D-galactoside	+	0	+	0
Methyl-β-D-thiogalactoside	0	+	+	0
Melibiose (α-galactoside)	0	?	+	0
Phenyl-β-D-galactoside	+	0	0	+C
Phenyl-β-D-thiogalactoside	0	+	0	+C
Phenyl-β-D-glucoside	0	0	0	0
Glucose	0	0	0	+ non C

* C = competitive.

A certain configuration of the molecule is thus necessary in order that it can be metabolized, another, in order that it can act as an inducer. To act either as a substrate or as an inducer of β-galactosidase, the substance has to be a galactoside. It may be said in general that the inducer is necessarily closely related to the substrate and has to share with it a characteristic chemical configuration. Here, as in many other cases, analogues may inhibit the specific effect of the inducer: the synthesis of β-galactosidase induced by methyl-β-D-thiogalactoside is inhibited by such substances as phenyl-β-D-thiogalactoside. The inhibition is specific and competitive (table 1).

As is well known, in some strains of bacteria the enzyme is formed only when an extrinsic inducer is present—the enzyme is said to be adaptive. In other strains, the enzyme is always there whether or not an inducer is present in the medium—the enzyme is said to be constitutive. The tests performed on β-galactosidase have shown that the adaptive and consti-

tutive enzymes have the same dissociation constant, the same thermal inactivation coefficient, and the same immunological properties. So far as can be judged, they are identical.

Apparently, the same protein is synthesized in one strain in the absence of extrinsic inducer; in the other, only in its presence. Does it mean that the intimate mechanism of the synthesis is different in each case?

In order to answer this question, data concerning sequential induction, as discovered by R. Stanier, have to be considered first. The carbon source of a bacterium, let us say glucose, is replaced by benzoic acid. It happens that the utilization of this substance involves a series of enzymes. The synthesis of the first depends on an extrinsic inducer, namely, benzoic acid. The product of the first enzymatic reaction induces the synthesis of the second enzyme, and so on. Adaptation involves here a series of inductions in which all the inducers, except the first, have been formed as the result of metabolism, that is, are endogenous (*2*).

Catechol oxidase is synthesized by some bacteria only if catechol is provided. But catechol is one of the end products of the metabolism of tryptophan. If tryptophan is provided instead, the bacteria will also form catechol oxidase (*3*). Although tryptophan is extrinsic, catechol is endogenous. If the bacteria could not dispense with tryptophan, tryptophan would have to be present in the medium, and it would be concluded that catechol oxidase is a constitutive enzyme. Another type of sequential induction is provided by yeasts in which oxygen induces the synthesis of a whole series of enzymes involved in respiration (*4*).

When we are dealing with such chains of reactions, the inducer of the last enzyme might be very different from the original substrate, which has induced the synthesis of the first enzyme. The original substrate is extrinsic. The last has been formed as a result of metabolism. If this metabolism deals with the synthesis of an essential metabolite, that is, it is not dispensable under experimental conditions, the inducer always would be present, and things would look as if the enzyme were constitutive.

It should be remembered: 1) that most enzymes belong to the constitutive group, 2) that the transition inducibility-constitutivity is controlled by a mutation, and 3) that most enzymes act on substrates that are synthesized by the bacterium itself.

Constitutive as well as adaptive enzymes may therefore depend on an inducer, even though the former are by definition formed in the absence of extrinsic inducer. If this is so, the substances that block the synthesis of adaptive enzymes should also block the synthesis of constitutive ones. This is the case: galactose inhibits the synthesis of β-galactosidase in adaptive strains and also prevents the synthesis in constitutive strains. The mechanism of this inhibition is not known, but the current hypothesis is that galactose competes with a natural endogenous inducer.

After considering all the data, M. Cohn and J. Monod (*5*) have proposed a generalized induction principle according to which the synthesis of all enzymes depends on specific inducers. The adaptive enzyme depends on an extrinsic inducer; the constitutive, on an endogenous one.

Unfortunately, we know very little about the way an inducer acts. From the study of induced synthesis of β-galactosidase, it was concluded (*1*) that bacteria must possess a specific induction site able to recognize the specific inducer, in this case a galactoside. This site, equivalent in specificity to the enzyme itself, is present and involved in induction, whether the inducer is extrinsic or intrinsic. Only bacteria which possess this induction site can produce the enzyme.

For the time being, the induced biosynthesis of enzymes is as difficult to visualize as the synthesis of any protein and will remain a matter of conjecture until we know more about the structure of enzymes and about the structural basis of their specific activity. The inducer could intervene within the folding or coiling of the peptide chain (*6*). It could also act in conjunction with the specific template, perhaps ribonucleic acid (RNA), which is supposed to be responsible for the organization of amino acids into peptide chains. Or it could act in conjunction with the specific deoxyribonucleic acid (DNA) carrying the information, which is sometimes supposed to be responsible for the production of a specific RNA template. Until we are faced with reality, it is agreeable to think that the specific induction site, which takes care of the inducer, has something to do with the gene.

Ignorant as we can be about the enzyme-forming system, some conclusions and hypotheses have been reached. The products of enzymatic activity might intervene as inducers in the synthesis of enzymes. Specific key sites should be involved in the synthesis of the enzymes as well as in enzymatic activity. The induction sites could be localized on the nucleic acid which is the carrier of information. Hypothetical as the key sites may appear, it is felt that they are of fundamental importance for the logical analysis and experimental approach to the problem of cellular correlations. Key sites will be postulated again and again in this discussion, especially in the conclusions.

Regulation of Enzymatic Activity and of Enzyme Synthesis

Everyone would probably agree with the statement that under given fixed conditions the constitution of a bacterium is constant. We have to conceive therefore of factors that regulate the synthesis of everything and in particular prevent one molecular species from outgrowing the others; that is, factors that prevent molecular cancer. Does the bacterium possess a "bureau of information" or an intelligence which is kept informed of what is happening everywhere in the protoplasm and which sends messages to stop or to activate such or such synthesis?

Analogues of the substrate of enzymes have been known for a long time to have the ability to inhibit enzyme activity; they compete with the substrate for the specific key site. And it is clear that the result of the activity of an enzyme, or of a chain of enzymes, is in general the production of structural analogues of the original substrate. Metabolism thus ends up in analogues which intervene in bacterial physiology in many ways, among which only two will be considered: regulation of the function-

ing of enzymes and regulation of the synthesis of enzymes. Before discussing the regulation of enzyme synthesis I would like to give two examples of functional enzyme regulation.

One of the end products in pyrimidine biosynthesis, cytidylic acid, is an inhibitor of the very first reaction in this chain of 6 enzymes. The result, as pointed out, is that if any surplus of nucleotide is built up it shuts off the synthesis of nucleotides and the process would thus be self-limiting. Woolley (*7*) has lucidly defined antimetabolites as "structural analogues antagonistic to metabolites." It is clear that cytidylic acid, which is a metabolite and even an essential metabolite, is also an antimetabolite. The existence of such dual functional activity of one substance is of fundamental importance.

When the growth rate of a bacterial population is reduced 10 times in a chemostat by a low level of tryptophan, there is no appreciable outpouring of arginine into the medium. This means that the rate of arginine synthesis is also reduced tenfold. The interpretation given by Novick and Szilard (*8*) is the following: When protein synthesis is slowed by a low rate of tryptophan supply, the internal "free amino acid level" of the other amino acid rises, and somehow the increased concentration of each amino acid depresses the rate of the individual steps of synthesis of this amino acid. Things happen as if the functioning of enzyme were inhibited by the substance resulting from their activity.

Let us now consider the problem of regulation of enzyme synthesis by the products of enzymatic activity. We have to come back again to β-galactosidase. When lactose is attacked by this enzyme, it is split into glucose and galactose. As stated previously, it was found that galactose, the result of β-galactosidase activity, inhibits the synthesis of β-galactosidase, and the question was naturally raised by M. Cohn and J. Monod as to whether or not this fact would be a particular expression of a general phenomenon; namely, the control of enzyme syntheses by the product of their activity.

The study of a few enzymes has been in agreement with the hypothesis. Methionine synthase catalyzes the reaction: serine+homocysteine→ methionine.

Escherichia coli grown in a synthetic medium without methionine contains methionine synthase; it would otherwise be unable to synthesize methionine. But if methionine is added to the medium and the bacteria allowed to grow for a few hours, it is found that they contain only 1 percent of the normal amount of methionine synthase. The synthesis of methionine synthase is blocked by methionine, which is a product of its activity (*5*).

Thus, a depression of the rate of synthesis of methionine synthase could be the result either of an increased production of methionine or of a decreased utilization of methionine. Any event which would modify the metabolism of methionine is therefore likely to produce a counter effect on the synthesis of the enzyme. This means that not only the enzymes taking part in the synthesis of methionine but also the totality of the

enzymes which control its utilization will take part in the regulation of the synthesis of methionine synthase.

A similar phenomenon has been disclosed for tryptophan desmase which controls the combination of indole and serine into tryptophan. The synthesis of tryptophan desmase is inhibited by both indole and tryptophan (*5*). Finally, it was also found that arginine inhibits the synthesis of acetylornithinase just as methionine inhibits the synthesis of methionine synthase (*9*). Acetylornithinase is an essential enzyme producing ornithine from acetylornithine on the pathway that leads to arginine.

We do not know whether the phenomenon is general, but we know that enzymatic activity generally results in the production of analogues. Therefore the hypothesis seems plausible that in many cases the control of the synthesis of the enzymes could be the result of a competition between an inducer and a competitor, the latter being the product of enzymatic activity as well as the inducer.

A substance which results from enzymatic activity may therefore possess two essential properties: *a*) it may be utilized as a building block for synthesis; *b*) it may act either as an inhibitor of enzyme activity, that is, as a regulator of a function, or as an inducer or inhibitor of enzyme synthesis, that is, as a regulator of growth. It is clear that the concentration of an analogue which results from enzymatic activity will depend on its further utilization, that is, on the activity of the whole enzymatic machinery.

In the generalized induction principle, the synthesis of any enzyme demands a specific inducer. Because enzymatic activity produces inducers, it is easy to understand why and how the synthesis of enzymes takes place as a result of metabolism. Because enzymatic activity produces inhibitors of the synthesis of enzymes, that is to say anti-inducers, it is easy to visualize how the synthesis of enzymes is stopped as a result of metabolism. A metabolite may act as an antimetabolite.

Let us assume that the generalized induction principle is wrong. That the synthesis of many or of most enzymes do not demand an inducer and all that is needed for the synthesis is a specific template and, of course, energy and building blocks. The building blocks for the synthesis of proteins, the amino acids, are, for all practical purposes, common to all proteins. The balanced synthesis of enzymes could be the result of a random distribution of amino acids on the different templates. If this is so all enzymes would be synthesized at the same rate and no regulation would be possible. If a regulation exists, a mechanism must be present that controls the syntheses specifically. The control might be positive—presence of a specific inhibitor—or negative—lack of a specific inducer. In any case a specific key site again has to be postulated. When this point has been reached, the principle of economy of hypotheses leads one to admit that only one key site is involved for both inducer and inhibitor. The regulation of the synthesis would be the result of the competition of an inducer and anti-inducer for a common receptor. It is only fair to

add that it has as yet not been possible to reverse the inhibitory effect of galactose on the synthesis of β-galactosidase by substances known to be inducers. According to the theory, this should be possible and the reason for the discrepancy between theory and experimental data is not clear. Possibly we do not know the "right" inducer. The problem of the real nature of the control of enzyme synthesis is far from being solved, but this should not undermine our conviction of the existence of a specific inducing site.

Permeases and the Problem of Drug Resistance

The result of enzymatic activity might be an inducer of the synthesis of an enzyme and an inhibitor of the synthesis of other enzymes. Its regulatory activity will, in the last analysis, depend on the metabolism as a whole. This in turn is controlled by the availability of a number of substances. As will be seen now, this availability is also controlled by mechanisms that depend on inducers.

It was known that some strains of *E. coli* which cannot utilize β-galactosides possess a β-galactosidase which is active in bacterial extracts. The enzyme is called cryptic. The hypothesis was put forward that crypticity is due to the fact that the substrate does not penetrate into the bacterium.

The study of situations pertaining to yeast and bacterial metabolism has led to the hypothesis that the penetration of various substrates inside a microbe could be something other than mere diffusion. The presence of active transfer mechanisms was postulated, which would be responsible for the transit across the membrane (*1*). The reality of such specific mechanisms has been demonstrated recently (*10*). It has been shown that the accumulation of β-galactosides in *E. coli* is controlled by a specific mechanism or structure. This structure is absent in bacteria that have not been in contact with a specific inducer. Its synthesis does not take place in the amino acid-exacting strains in the absence of any of the required amino acids. It is inhibited by chloromycetin and thienylalanine. The analogue of β-galactosides that inhibit the synthesis of β-galactosidase generally inhibit the synthesis of the structure. Its kinetic properties, for example, its steric specificity as a transporter, are those of an enzyme. Its functioning, that is, the intrabacterial accumulation of galactosides, depends on reactions giving metabolic energy. Finally, the possibility of its synthesis is controlled by mutations that are distinct from those that control the possibility of synthesizing β-galactosidase. The "mechanism" is thus probably protein in nature and is possibly and probably an enzyme. It was called *permease* by Rickenberg, Cohen, and Monod.

Other experiments have brought the proof that the transfer of many sugars, such as glucose, and of the substrates of the Krebs cycle across the bacterial membrane also depend on induced enzymes (*1*).

The existence of permeases allows us to interpret data concerning the physiology of amino acids. It was found, for example, that isoleucine

blocks neither growth nor incorporation of endogenous valine into the proteins. But isoleucine prevents the incorporation of radioactive extrinsic valine. This is probably because it blocks its penetration as the result of the inhibition of the activity of a specific constitutive permease (*11*). And it seems as though specific enzymes would also control the accumulation of groups of structurally related amino acids.

According to the conclusions of Rickenberg, Cohen, and Monod, permeases appear to be systems of a protein nature which are responsible for the catalytic transfer of many substances across the bacterial osmotic barrier. As far as we know permeases possess the steric specificity and the kinetic activity of enzymes. And they are distinct and independent of the enzymes responsible for the metabolism proper of the substrate.

Thienylalanine, an analogue of phenyalanine, inhibits the growth of many bacteria. This is the case in the wild type of *E. coli*. Thienylalanine is accumulated into the sensitive bacteria, whereas resistant mutants, as shown by G. N. Cohen (*12*), are unable to accumulate the inhibitor. Here, the resistance to a drug is the result of the loss of the mechanism responsible for the transfer of the inhibitor across the cell membrane.

Sensitivity or resistance to a drug is, thus, in some cases controlled by the presence or absence of a permease—or in more general terms depends on the accessibility of the specific enzyme system to a drug. This system is believed to be identical with the one that concentrates the normal essential metabolite.

Memory

Essential metabolites and their analogues, inducers and anti-inducers, appear as essential factors in growth and growth regulation. Metabolism is the result of the specific structure of the organisms and of the functioning and interplay of its enzymes, the synthesis of which depends on inducers. When the inducer of an enzyme is suppressed, the synthesis of this enzyme ceases.

The alteration produced by an inducing agent is not inheritable. However, it was discovered by M. Cohn (*13*) that a short initial stimulus can induce a functional change which can be maintained indefinitely. This phenomenon is important for it could account for many situations encountered in cells of higher organisms.

First we have to recall that glucose is able to inhibit the synthesis of galactoside permease as it inhibits the synthesis of a number of enzymes dealing with the metabolism of amino acids, respiration, etc. This nonspecific inhibition, which is not competitive, is for the time being not understood.

Let us now consider a bacterium that can synthesize β-galactosidase and the corresponding specific permease and in which the synthesis of both enzymes demands an extrinsic inducer. These bacteria are grown in a medium containing maltose. No enzymes for the metabolism of β-galactosides are present. If glucose is added and afterward a β-galactoside,

no enzyme will be produced either. Glucose blocks the synthesis of the permease and, as a result, the synthesis of β-galactosidase.

If the β-galactoside is added first and glucose only after 10 minutes, things are different. A few molecules of the β-galactoside have crossed the bacterial wall by diffusion. This process is very slow, but enough molecules have managed to penetrate to induce the synthesis of the permease. When glucose is added after 10 minutes, each bacterium possesses a few molecules of permease. As a result of its activity, β-galactosides are pumped into the bacterium. And the amount which is pumped can victoriously compete with glucose for the enzyme-forming system. If the concentration of glucose is well chosen and severely controlled, a bacterium which has lived in the presence of a β-galactoside for a third of a generation will be able to synthesize both permease and β-galactosidase in the presence of glucose for at least 133 generations. This means that the induced state has remained unchanged when around 10^{40} microbes, that is, 3×10^{21} tons of bacteria have been produced out of 1 bacterium, or out of 3×10^{-10} mg. It can safely be concluded that a steady state has been reached.

Here, the presence of a set of enzymes, namely β-galactoside permease and β-galactosidase, depends on whether the bacterium has been in contact with one substance, the inducer, before having been in contact with the other, the inhibitor.

If glucose were a necessary constituent of the medium and a β-galactoside always available to the bacteria, both β-galactoside permease and β-galactosidase would be present and considered as constitutive enzymes. If, for one reason or another, β-galactosides were unavailable for a period, both enzymes could disappear forever under experimental conditions—and it would appear as if a structure endowed with genetic continuity had been lost.

The specific information, an energy source, essential metabolites, and a specific inducer are necessary but not sufficient for the synthesis of a specific protein. The inhibitor can compete with the inducer, and, as seen before, a flux equilibrium can be involved in determining the subsequent state and balance of the synthesizing system. The synthesis of an enzyme thus depends on the interplay of the metabolites as controlled by the *milieu intérieur*, that is, of metabolism, which in turn is controlled by the enzymes and by the composition of the culture medium.[3] This interplay, as will be discussed, is really very intimate and intricate.

Amino Acids and the Synthesis of Nucleic Acids

It is generally admitted that nucleic acids have something to do with protein synthesis. This is certainly so (*16*). The theories concerning the

[3] A steady state could also be involved in higher organisms in modulation or differentiation. The latter is currently defined as an irreversible change of potentialities (*14*). A cellular change which appears permanent under natural or experimental conditions can be, but is not necessarily, bound to an irreversible alteration or to the formation *de novo* or loss of a particle endowed with genetic continuity. It could be the result of a steady state imposed by a characteristic of the medium which would also be necessary for the maintenance of the cell. It has been stated (*15*) that although differentiation is irreversible, irreversibility increases with the degree of differentiation. This seems to mean *a*) that differentiation is reversible, and *b*) that the degree of reversibility is variable.

role of nucleic acids were reinforced when it was conceived that genes control enzyme synthesis and that specific information for the synthesis of specific proteins is contained in nucleic acids. The experiments aiming to prove the direct intervention of nucleic acids in protein synthesis have failed so far. Something quite unexpected turned out, however, namely, that the absence of one amino acid was enough to prevent nucleic acid syntheses.

Deficient strains of *E. coli* are known, which are unable to synthesize various amino acids. It was found that upon specific starvation of the nonsynthesized amino acid, the synthesis of DNA and RNA was stopped (*17*, *18*). The interaction between amino acid and the synthesis of RNA was investigated further by F. and F. Gros (*19*), who have confirmed and really proved their necessity for RNA and DNA synthesis and thoroughly analyzed and discussed the role of tryptophan and of phenylalanine. Some 1,000 molecules of phenylalanine per bacterium are enough for the synthesis of nucleic acid at maximal rate when the synthesis of proteins is blocked. Moreover, free phenylalanine is almost completely recovered at the end; the action of the amino acid is therefore catalytic. Nine amino acids so far have proved essential: leucine, valine, proline, ornithine, threonine, histidine, tryptophan, phenylalanine, and methionine,[4] and the problem of their mode of action was naturally raised. The hypothesis has been put forward that, in the synthesis of nucleic acids, amino acids would act as transporters of a sequence of nucleotides (*18*, *19*). This hypothesis is in agreement with prevailing theories concerning the role of nucleic acids in the synthesis of proteins and according to which a group of nucleotides takes care of one amino acid.

Bacteria are thus unable to synthesize nucleic acids in the absence of amino acids. As is well known, both RNA and DNA can be synthesized *in vitro* in the absence of free amino acids. The apparent contradiction between bacteria and biochemists will have to be resolved. The only thing that should be noted is that the nucleic acids synthesized by biochemists are certainly different from those produced by bacteria.

Incorporation of Analogues

Growth of a bacterium can be inhibited by a variety of analogues. An analogue of a substrate or antisubstrate of an enzyme can inhibit the functioning of this enzyme by competing with the normal substrate. An analogue of an inducer, or anti-inducer, can inhibit enzyme synthesis by competing with the inducer. Specific key sites are necessarily involved, because if competition takes place, it has to take place somewhere for something. A key site is a part of a high molecular structure. The specificity of this high molecular structure, whether a protein or a nucleic acid, is, in the last analysis, the result of the sequence of its building blocks. Here again analogues of building blocks might inhibit bacterial growth by interfering with the incorporation of normal building blocks.

[4] This could perhaps explain why ribonucleic acid-soluble fragments are excreted as a result of methionine starvation (*20*).

Although very little is known about abnormal syntheses, they will be briefly considered, for they are an important aspect of the problem of cellular growth and cellular disease.

I apologize for having to refer once more to β-galactosidase. A short digression on the dynamic state of cell structure is necessary. An extensive study of the β-galactosidase in intact, growing *E. coli* has not allowed to detect any renewal of the sulfur of the enzyme. Whether the β-galactosidase did function or not, the protein was not measurably dynamic.[5] All experiments that aim to show a replacement of phosphorus or of purine and pyrimidine carbon of the DNA in growing bacteria have failed so far. And it was impossible to detect a flow of phosphorus atoms from RNA to DNA. This means that structures like protein or nucleic acids, despite an intense functional activity, behave as structural wholes. Building blocks are incorporated into protein and nucleic acid only when syntheses are performed. We are interested to know if analogues of building blocks, that is, anti-building blocks, are also incorporated if abnormal syntheses are performed in their presence, and if anti-building blocks take the place of normal building blocks, that is, if there is something like a competition for a receptor. We are, of course, also interested to find out how the analogue, considered here as an anti-building block, compares with analogues of substrates, which block enzymatic activity, and with analogues of inducers, which block the synthesis of enzymes. Data concerning these problems are only beginning to be available.

When thymine is withheld from the strain of *E. coli* which cannot synthesize thymine, synthesis of DNA stops, but synthesis of RNA and of protein continues for a time and within an hour 99 percent of the bacteria die. The same bacteria will survive in the absence of a carbon and energy source, if no synthesis can take place (*21–23*). Death seems to be the result of the unbalanced synthesis. Recently, however, it was found that the nucleic acid of many normal bacteria contains 6-methylaminopurine (*24, 25*). In the thymineless strain of *E. coli*, as a result of thymine starvation, 6-methylaminopurine accumulates and seems to take the place of thymine (*25*). The hypothesis was put forward that death of the thymine-starved bacteria is the result of the incorporation of an analogue of thymine. This is probably the mechanism of the killing effect of an analogue of thymine such as azathymine. When azathymine is added to exponentially growing *Streptococcus faecalis*, bacteria continue to grow but about 50 percent die within about 2 hours. In the DNA of such bacteria, 12 to 18 percent of the thymine is replaced by azathymine (*26*).

In one case, where thymineless bacteria are concerned, thymine is replaced by an analogue, 6-methylaminopurine, which is synthesized by

[5] As pointed out by J. Monod, the experiments performed on bacteria do not mean that all proteins are stable in all cells at all times; they mean simply that "dynamicity "of cell constituents, if it exists, is not general.

Cell constituents are sometimes referred to as "living matter." However, none of the cellular structures considered separately from the whole possess the properties of a cell. Life is the privilege of organisms and of cells which are organized totalities. A macromolecule, when called "living," is, by virtue of a word, unconsciously endowed with the attributes of life, and the result is a great disorder. It should be borne in mind that such a thing as "living matter" does not exist.

the bacterium and is a normal constituent of DNA. In the other case, thymine is replaced by the analogue, azathymine, which is extrinsic and has nothing to do with the normal metabolism. In both cases, analogues of thymine have taken the place that would have been normally occupied by thymine; a specific receptor or niche for thymine and its analogue has to be postulated.

It is known that some analogues of purine and pyrimidine bases can increase considerably the mutation rate of bacteria (*27*) and of bacteriophage (*28*). And there is little doubt that this is due to their incorporation into nucleic acid, which results in an altered genetic structure.

8-Azaguanine, the analogue of guanine, is known to inhibit the growth of many microorganisms and viruses and to be incorporated into their nucleic acid. When *Bacillus cereus* has for a time performed syntheses in the presence of 8-azaguanine, the analogue is found in RNA and DNA. The percentage of 8-azaguanine which replaces guanine is 25 percent for RNA and 1 percent for DNA. Moreover, 8-azaguanine is not incorporated at random in RNA but preferentially in the end group of the original nucleic acid (*29*). The significance of this important observation is yet somewhat cloudy.

From these observations, it can be safely concluded that when a bacterium performs syntheses in the presence of analogues of purine and pyrimidine bases, the analogues are incorporated in lieu and place of the normal substances. As will be seen, this conclusion is valid also for analogues of amino acids.

When *p*-fluorophenylalanine is added to *Escherichia coli*, the bacterial mass, which has been increasing exponentially, now increases linearly as a function of time. The synthesis of protein is also a linear function of time as shown by the kinetics of the incorporation of labeled methionine and valine. The analogue *p*-fluorophenylalanine is also incorporated. The proteins which are synthesized in its presence contain less phenylalanine and tyrosine than the controls, whereas their content in valine + isoleucine is the same. Moreover, the bacteria excrete phenylalanine and tyrosine. So *p*-fluorophenylalanine partially replaces phenylalanine and tyrosine. The fraction of the 2 amino acids synthesized by the bacteria which is not utilized for the synthesis of proteins is excreted (*30*). Other analogues, namely, β-2-thienylalanine or leucine, are also incorporated into proteins when added during the exponential growth phase.

The question was naturally raised of the functional activity of the proteins in which analogues have been incorporated. The proteins formed in the presence of thienylalanine are devoid of β-galactosidase activity. In the presence of *p*-fluorophenylalanine, β-galactosidase is synthesized, whereas galactoside permease activity does not increase (*30*); as a result, a situation functionally identical to crypticity is produced. The actual presence of *p*-fluorophenylalanine in the β-galactosidase has not yet been demonstrated but seems probable. A new way is thus open for the study of the effect of various building blocks of a protein on its activity as an enzyme and should allow us to learn something about the anatomy of the key site.

A number of experiments have thus shown that bacteria die when allowed to perform syntheses in the presence of analogues of building blocks of nucleic acid or of protein. The time between the initiation of the pathological condition and death will be a measure of the "killing efficiency" of a drug. It depends on many factors and in particular on the relative affinity of the analogue and of the normal metabolite for the alleged receptor and whether or not nucleic acid and, especially, the genetic material is involved in the abnormal synthesis.

Unbalanced Growth

Growth can be abnormal because the synthesis of one enzyme is inhibited or because anti-building blocks are incorporated into essential structure. Abnormal growth can also be the result of the preferential or exclusive development of one type of substance or even of the development of a new substance (*31*, *32*).

When a culture is supplemented with chloromycetin, bacteria continue to grow. They do not synthesize proteins but only nucleic acid (*33*). As a result, the ratio of protein to nucleic acid becomes more and more upset. If the contact is long enough, bacteria die. If the contact has been short, bacterial multiplication will be resumed, but it will start only after a few hours when the normal condition has been restored.

Bacteria partially deprived of amino acids can synthesize RNA without synthesizing DNA or protein. When normal external conditions are restored, these bacteria remain for 2 hours unable to synthesize proteins and, in particular, our old friend β-galactosidase (*17*). This will not surprise anyone.

Finally, it should be added that bacterial growth can be upset not only by analogues which induce the formation of abnormal structures and by inhibitors which induce an unbalanced molecular development but also by factors that induce the formation of completely new structures, which may or may not be infectious. Some bacteria, called bacteriocinogenic, perpetuate the property to synthesize lethal proteins called bacteriocins. These substances are not normally present in the bacterium. They may be produced as a result of the action of factors or substances that are all known to be mutagenic or carcinogenic or both (*31*, *34*). The bacteriocinogenic bacterium then starts synthesizing bacteriocin and dies as a result of this synthesis. Other bacteria, called lysogenic, perpetuate the power to synthesize phage material and phage particles; this synthesis is also lethal. Both properties are perpetuated in the form of a gene-behaving structure (*35*). Viruses, and in particular phage, block the synthesis of the host material and induce the synthesis of new material, namely virus structures. In this respect, virus development is one of the aspects of unbalanced growth, the pathological aspect of the synthetic process which results in infectious particles. This is, however, collateral to collateralism.

For the time being we might conclude that disease and death of a bacterium can be caused either by an unbalanced development of a given

molecular species or by the production of an abnormal molecule. Unbalanced development is the production of one structure in excess; it is clear that this process can produce disorganization of the internal architecture and thereby functional disturbances. Production of abnormal molecules may affect enzymes or nucleic acids; the end result, as far as survival is concerned, will depend on the nature and function of the structure involved and on the extent of the abnormality.

Discussion and Conclusions

Physiological growth of a microorganism corresponds to the balanced synthesis of normal structures—including, of course, the duplication of the genetic material. Physiological growth results in multiplication.

A bacterium is an orderly system in which enzymes and genetic material cooperate for the production of two identical systems. Neglecting the mechanisms that produce energy, growth might be described as the result of two processes, *a*) synthesis of essential metabolites, *b*) organization of essential metabolites into specific structures such as proteins and nucleic acids.

Physiological growth may be upset by a variety of events, factors, or conditions. These can be as different, for example, as the development of bacteriophage which abruptly induces syntheses of virus material and the action of abnormal metabolites which block enzymatic activity or induce abnormal syntheses. The development of bacteriophage is always lethal; the disease caused by the action of abnormal metabolites is lethal only if their action lasts long enough. Survival of a vegetative bacterium depends on physiological growth, and any metabolism which does not lead to, or end up with, reproduction may be considered pathological.

The penetration of many organic substances into the bacterium as well as the synthesis of the building blocks depends on enzymes. The organization of building blocks into specific enzymes depends on specific information carried by the genetic material. For the synthesis of a protein all "biological" amino acids have to be present. For the synthesis of nucleic acid, which is the carrier of information, all "biological" purine and pyrimidine bases and also all amino acids, acting here as catalysts, must be available. Moreover, *a*) the backbone of purines is formed from a molecule of glycine, *b*) aspartic acid is involved in the synthesis of pyrimidines, and *c*) amino acids or purines and pyrimidines nucleotides are constitutive parts of many coenzymes.

It is essential to remember that the synthesis of an enzyme dealing with the metabolism of an amino acid demands amino acids—the common building blocks of the proteins—a specific nucleic acid, and, in many cases, a specific inducer. If the inducer comes from outside, it has to be pumped into the bacterium by a specific enzyme. If the inducer is synthesized, it is through a chain of enzymes. The end product of a chain of reactions can act as an inducer of the synthesis of an enzyme belonging to this chain and as an inhibitor of the synthesis of another enzyme of the same chain.

An inducer might also be an anti-inducer, and a metabolite might also be an antimetabolite.

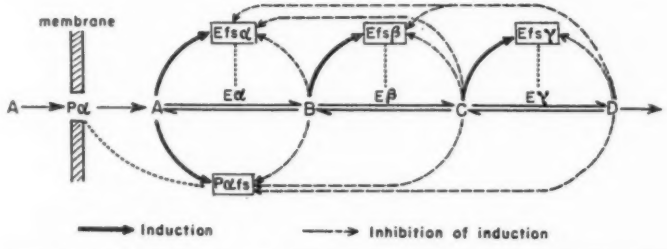
An amino acid is therefore a building block for the synthesis of enzymes, a substrate for their activity, and may be an inducer of the synthesis of some enzymes and an inhibitor of the synthesis of others. Amino acids are also necessary cogs in the synthesis of nucleic acids. These, in turn, are the carriers of the specific information, the controllers, and may be the templates for the synthesis of enzymes.

Let us assume that the induction site of the enzyme-forming system is part of a nucleic acid template. An amino acid, whether it is a building block, a substrate, an inducer, or a cog, has to be able to combine with an enzyme and with a nucleic acid. This is possible only if both enzyme and nucleic acid possess a homologous receptor or key site. Homologous means, here, sterically equivalent or related, although structurally and functionally different. It is clear from the data computed in table 1 that homologous receptors do not deal with identical structures but with certain types of structure, and that homology does not imply sterical identity.

From the classical data concerning the inhibition of enzymes by analogues of the substrate and from the data discussed here concerning inhibition of the synthesis of enzymes and incorporation of analogues it is clear that antimetabolites can be considered functionally under 3 headings: 1) antisubstrates—they compete with the substrates and inhibit enzymatic activity; 2) anti-inducers—they compete with the inducers and inhibit enzyme synthesis; 3) anti-building blocks—they compete with the building blocks and enter the constitution of structures which, as a consequence, are abnormal.

The antagonism between metabolites and antimetabolites implies a competition for a common receptor or key site.

Considered from a functional point of view, key sites belong to 2 different categories: *metabolic key sites* responsible for enzymatic activity, and *structural key sites* responsible for the organization of building blocks, that is, for the specific synthesis of proteins or of nucleic acids. Key sites have been postulated in order to account for the competition of metabolites and antimetabolites. Pairs of homologous receptors must be



TEXT-FIGURE 1.—A, B, C, D = substrates; Eα, Eβ, Eλ = enzymes; Efs = enzyme-forming system; Pα = permease for substrate A; Pαfs = permease-forming system. Induction and inhibition of induction might involve one or more enzymatic reactions.

postulated in order to account for the dual interaction of a given substance with both a metabolic and a structural key site.

The conclusion must be drawn that the activity of metabolic key sites produces substances which control the activity of structural key sites. Pathology aside, metabolism, growth, and reproduction appear as self-regulating processes in which the products of enzymatic activity control the synthesis of enzymes.

In a straight chain of enzymatic reactions the product of one enzyme is the substrate for the next. In a sequential induction, the substrate of each enzyme is the inducer of the synthesis of this enzyme and the inhibitor of the next. A model was discussed by M. Pollock (*41*) in which the cell is regarded as an integrated system of successive inductions; I would propose to add, "and inhibitions." This functional definition has to be completed by the introduction of anatomical features, and a cell could accordingly be described as an organized and balanced system of integrated structures and syntheses.

We have to conceive, were it only as a working hypothesis, some sort of mechanism of information and feed back by which nucleic acid, as controller and regulator of specific syntheses, is being informed of what is going on in the protoplasm and directs metabolism in consequence.

From the data and hypotheses which have been discussed, the idea has developed that the essence of balanced growth could be the interplay of metabolites with pairs of homologous receptors. We begin to understand why and how metabolism controls the synthesis of both the structural and the metabolic key site, or, in other terms, why and how structures and metabolisms cooperate for the command and control of growth and reproduction.

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DISCUSSION

Dr. Woolley: I thought because of Dr. Lwoff's excellent presentation that I could do no better than to add another facet to the picture. As a result of experiments conducted in plant tissue culture, in living animals, and in bacteria, a new phenomenon seems to be emerging. This phenomenon is that differentiated cells differ from corresponding, less-differentiated cells in having lost the ability to synthesize certain essential metabolites. Let me illustrate this, first, in connection with animals. Experiments which we have conducted during the past 6 years have shown that vitamin B_{12} (abbreviated structure shown in text-fig. 1) is not synthesized by normal animals but is synthesized by those bearing spontaneous mammary cancers. May I illustrate this point with a few data. This phenomenon has been shown in 2 ways. First of all, one can measure the effect of the cancer on the synthesis of B_{12} in the mother of a litter of mice. Merely by measuring the growth rate of the offspring, one can determine that in the cancerous mother B_{12} has been formed, whereas in a noncancerous animal this is not the case (table 1). These animals were all kept on vitamin B_{12}-deficient diets. The litters from cancerous mothers kept on these diets grew faster because of the production of vitamin B_{12} in the mother, who is the host to the cancer. You can also demonstrate this fact by merely analyzing the mother mice for vitamin B_{12}. Table 2 shows the amount of vitamin B_{12} in mice fed a vitamin B_{12}-deficient diet; those with cancers had more vitamin B_{12}.

The less-differentiated tissue, the cancer, synthesizes the vitamin which must be supplied from outside for the normal tissues. Having found this, we recalled that Dr. Gautheret, many years ago, had demonstrated an identical phenomenon in the case of crown gall in plants. There, as you all know, it was shown that, whereas the normal tissue did not synthesize indoleacetic acid, the cancerous tissue, the gall, did. Here then is the same phenomenon. The less-differentiated tissue is performing a synthesis which the normal more-differentiated tissues do not. There is a third case which has recently appeared and which will be discussed in much more detail by Dr. Braun. This case was first found, I believe, by Dr. Steward, when he found the requirements for the coconut-milk factor in the growth of certain kinds of carrot-root cells. Dr. Steward pointed out that the cambial cells of carrot root were able to grow on a synthetic medium, as had been known before, but that non-cambial cells required something that was produced in the cambial cell and was contained in such things as coconut milk. Here again we see the same phenomenon: the less-

TABLE 1.—*Growth rate of mice from normal and tumorous mothers*

Mothers	Rations fed the young	Animals	Average gain *(gm./week)*
Normal	B_{12}-deficient	33	3. 4
Tumorous	B_{12}-deficient	49	4. 5
Normal	B_{12}-deficient, but mothers fed B_{12}.	16	4. 9
Tumorous	Plus B_{12}	7	5. 4
Normal	Plus B_{12}	24	5. 2

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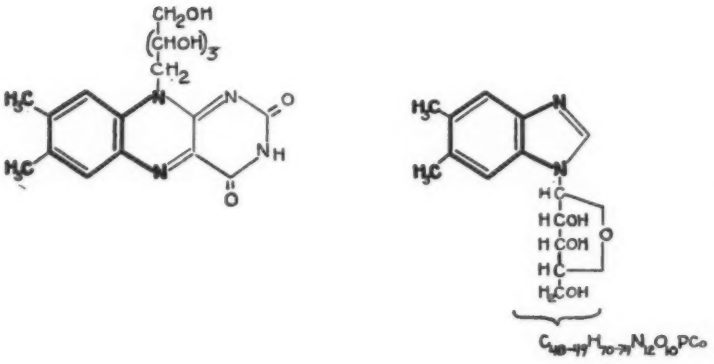


TEXT-FIGURE 1.—Structure of vitamin B_{12}.

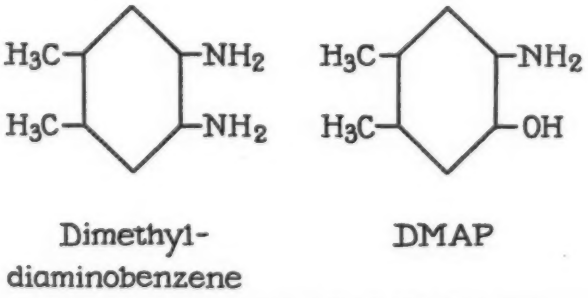
TABLE 2.—*B_{12} in mice maintained for many weeks on B_{12}-deficient ration**

Condition	No. of mice	Avg. wt. (*gm.*)	Avg. B_{12} content (*μg./gm.*)
Normal	18	28	9. 5
Cancerous	15	31	17. 2

*Swiss mice bearing *spontaneous* mammary cancers when started on the deficient ration.

differentiated cell, the cambial cell, carries out the synthesis which the more-differentiated ones do not. With these 3 examples from widely separated and perhaps collateral fields, we begin to see the possible emergence of this new phenomenon.

There is some practical value in this sort of discovery. In connection with the mammary cancers, for example, a working hypothesis had been built up on how one could cure them, if one produced an antimetabolite of the precursor from which vitamin B_{12} was being formed. This precursor is dimethyldiaminobenzene (text-fig. 2) and is a common precursor of both riboflavine and vitamin B_{12}. Reference to text-figure 1



TEXT-FIGURE 2.—Structure of dimethyldiaminobenzene.

will make this clear. It was demonstrated that dimethyldiaminobenzene did in fact act as a precursor in living cells for these 2 vitamins. The problem is to make an antimetabolite of dimethyldiaminobenzene such as DMAP, the structure of which is shown in text-figure 2. This antimetabolite should not harm the normal animal because the normal animal does not synthesize the vitamins. It should harm the cancers and, in fact, it has been possible to show this. By making suitable analogues of dimethyldiaminobenzene, it has been possible to cure permanently a small percentage of spontaneous mammary cancers in mice, and we hope that, when we have more active antimetabolites of this series, it may be possible to cure all of them.

Dr. Thimann: I feel that Dr. Lwoff has done, perhaps, a rather notable thing in attempting for the first time, to put together a complete picture of the balanced growth of an organism viewed in a mechanistic way. In order to do this, of course, he has had to make a number of proposals. Some of them are obviously acceptable, some, perhaps, less so.

The first important proposal is that the synthesis of all enzymes is essentially induced. I think that this idea may well be justified. It is difficult to marshal any heavy evidence against it. The only serious difficulty, it seems to me, may be encountered in the *de novo* synthesis of enzymes such as we see in the germination of bacterial spores. There are, as you know, a number of bacterial spores that will germinate in distilled water; some others will germinate in very simple solutions like alanine. Now the work of Powell, Hardwick and Foster, Cook, and others, has shown that many bacterial spores are essentially devoid of enzyme activity. It is difficult to demonstrate a single live enzyme in them. Furthermore, a quantity of their organic nitrogenous material, which would be used in the synthesis of enzymes, is diverted to other substances like glucosamine and dipicolinic acid, which are simply excreted when the spore germinates and are apparently of no further use to the cell. It is very difficult to see, under such circumstances, how the induction of the entire enzyme battery of the living cell could take place on germination. There are, of course, a number of difficulties in connection with the role of analogues in inhibiting the synthesis of enzymes which Dr. Lwoff presented so persuasively. But we do see that an analogue does not necessarily inhibit the synthesis of an enzyme and indeed very often enzyme synthesis is inhibited by related materials that are not the enzymatic products. Perhaps a notable example is the tryptophan-synthesizing enzyme, which is inhibited not by the product so much as by indole, which, after all, is one of the two substrates. It is difficult to see how such an inhibition by substrate could play any part in the control of enzyme formation by the products.

In Dr. Lwoff's discussion of the permeases, we are perhaps on still more shaky ground. It is true, of course, and has often been observed, that the uptake of an organic compound may be inhibited by analogues. In *Neurospora*, for instance, the uptake of leucine is inhibited by δ-chloroleucine, the uptake of lysine is inhibited by arginine, while the uptake of histidine is inhibited by several amino acids and, very powerfully, by mixtures of two; one amino acid plus arginine or leucine. We should note, too, that this inhibition of uptake is not limited to those organisms that are dependent on an external source of the amino acid. It is mainly observed there because their growth, which is proportional to the uptake, becomes inhibited in consequence. But Matheson and Catcheside, last year, studied the wild type *Neurospora*, which is not dependent on the uptake of histidine because it synthesizes histidine, and showed that in this organism the accumulation of histidine from outside is still inhibited by the analogues. Evidently, therefore, such inhibition is a common phenomenon, and the question is: What is the justification for invoking specific uptake enzymes for this purpose? We should note, first, that this inhibition is not necessarily competitive. Perhaps the best example of this is in Gale's studies of the uptake of glutamic acid. You will remember, this is inhibited by cysteine but the inhibition is not of the competitive type at all. Instead, curiously enough, glutamyl cysteine is actually synthesized outside the cell and is found in the medium.

It may be worthwhile to think for a moment in more general terms. Cells can be experimentally exposed to all kinds of materials and are able to absorb foreign com-

pounds, which they never would encounter in the course of a normal life. All sorts of drugs can be administered to cells and can be taken up. Are we to deduce that cells have enzymes for all kinds of compounds, which they normally would never encounter? It is hard to believe. More probably the uptake of these foreign and unnatural substances is somehow organized by the systems that are there for taking up natural substances, and so we have to suppose that a wide variety of synthetic compounds can somehow get in "on the coattails" of the specific normal substrates for which there are enzymes (assuming these enzymes exist at all outside of the cell). Then, too, uptake is not limited to organic compounds. The accumulation of solutes against a gradient takes place and, perhaps, most strikingly, as far as plants are concerned, with inorganic ions rather than organic compounds. There are famous cases of plants which accumulate potassium so that the internal concentration is 1,000 times the external. Are we to suppose that there are special uptaking enzymes, permeases, for every inorganic ion? Since plants distinguish between different ions, they would have to possess separate enzymes for each ion. Ions, which are very rare in nature, such as rubidium and caesium are readily taken up. It seems to me the only way out of this is to conclude that permeases would have to be highly nonspecific, otherwise they could not function at all. And if they are highly nonspecific then their value in explaining these phenomena is in good part lost.

These thoughts that Dr. Lwoff has put forward are, however, very interesting in another respect, namely, with regard to growth-promoting substances. It may be true that the very delicately balanced growth in a bacterium is not influenced by growth-promoting substances, but in plants, where growth is not nearly so delicately balanced, and one process can run ahead of another, auxins and other growth-promoting substances are clearly in control of growth. One wonders how an auxin could possibly act in a balanced system, such as Dr. Lwoff has described, where amino acids control the formation of ribonucleic acid and then are formed by this ribonucleic acid into proteins under the control of the very proteins that have been formed, so that too much protein does not accumulate. It has been one of the puzzles of the study of plant growth substances that it is difficult to characterize the first step. It can be shown that, in general, the hormone evidently acts on some limiting factor, such as those properties of the cell wall that limit elongation, or the specific factors limiting cell division. May one, perhaps, go beyond Dr. Lwoff's remarks to suggest that the growth substance acts on ribonucleic acid in the cell that forms the enzymes?

Dr. Vennesland: To an enzymologist, the facts that Dr. Lwoff has summarized appear as a collection of biological phenomena in search of chemical reaction mechanisms. The time may not be far off when we will understand the chemical events that underlie the process of induced enzyme synthesis. Perhaps the most fertile current approach to this problem is by way of the investigation of the enzymology of the nucleotide derivatives of carbohydrates and amino acids. I shall not elaborate on this subject, however, since it is not the field in which I have been working.

Let me make just two specific comments on Dr. Lwoff's paper. The first concerns a question of semantics in regard to the use of the word permease. The structure of this word implies that permease is a protein that catalyzes a penetration or transfer of a material from the exterior to the interior of the cell. The most familiar analogy, which comes to mind, might be that of hemoglobin, which could be called an oxygen transportase. You may, of course, define permease in any way you wish, but I wonder whether there isn't a danger here in the sense that the word implies a knowledge of mechanism which we do not have.

Then I should like to comment on the interesting hypothesis that all enzymes may be induced by their substrates. In this connection, one cannot help recalling an analogy which has been pointed out frequently by others. This is the analogy between adaptive enzyme formation and antibody formation. Within the framework of this analogy, and of the aforementioned hypothesis, the substrate of an enzyme, which, we will assume, acts as an inducer, appears to resemble a hapten. A hapten, however, doesn't induce antibody formation unless it is combined with a protein. Can we find a protein with analogous function in the induction phenomenon? I

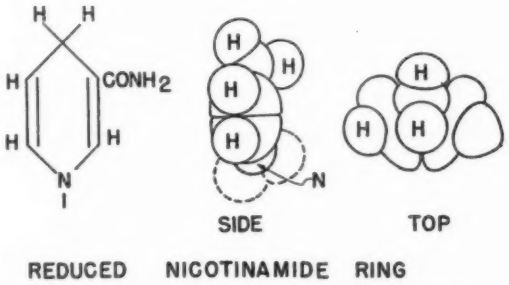
would like to suggest that such a protein may be available in the form of the enzyme that catalyzes the preceding enzyme reaction. (*See* text-fig. 1 in Dr. Lwoff's paper, p. 525.)

Let me illustrate this possibility with a specific example. The reaction catalyzed by D-glyceraldehyde-3-phosphate dehydrogenase proceeds according to the equation:

$$\text{triosephosphate} + \text{phosphate} + \text{DPN}^+ \rightarrow \text{1,3-diphosphoglyceric acid} + \text{DPNH}$$

(In this equation DPN^+ represents oxidized diphosphopyridine nucleotide, and DPNH represents reduced diphosphopyridine nucleotide.)

All of the reaction components may be considered to be substrates of the enzyme. With the help of deuterium as a tracer, my associates, Richard Levy and Frank Loewus, have shown that when triosephosphate reduces DPN in the presence of triosephosphate dehydrogenase, a hydrogen atom is transferred directly from the triose to the nicotinamide moiety of the DPN. In text-figure 1, the structural formula on the left represents the reduced nicotinamide ring of DPN, as we ordinarily write it. At the *para*-position, opposite the nitrogen, are 2 hydrogen atoms. In lactic acid fermentation, the DPN is reduced by triosephosphate, so 1 of the 2 hydrogen atoms will have been donated by triose in the presence of triosephosphate dehydrogenase. Then this reduced DPN is reoxidized by pyruvate in the presence of lactic dehydrogenase, in which process a hydrogen atom is transferred from the reduced DPN to pyruvate. But the hydrogen atom which is transferred to pyruvate is not the same hydrogen atom which was previously transferred to DPN from triose. In the reduction by triose, hydrogen is transferred to 1 side of the ring only. Then, in the reoxidation, hydrogen is removed from the other side of the ring.



TEXT-FIGURE 1.—Reduced nicotinamide ring.

Examination of the geometry of the reduced nicotinamide ring, with models, makes it simpler to visualize what is occurring. Text-figure 1 shows 2 drawings of molecular models, 1 looking at the reduced nicotinamide moiety from the side (away from the carboxamide substituent), the other looking down on it from the top. We are particularly interested in the position of the 2 hydrogen atoms at the *para*-position. Note that 1 of these lies in the same plane as the hydrogen substituents on the other carbon atoms, in what we may call an equatorial position. The other *para*-hydrogen atom protrudes from this plane, to the right, in an axial position. It is the axial hydrogen atom that is probably transferred in the dehydrogenase-catalyzed reaction. The point to be emphasized is that both of the hydrogen atoms may occupy an axial position though not simultaneously. The drawing represents only 1 of the configuration of the ring, *i.e.*, 1 of the ways in which it may be folded. The figure shows a hydrogen atom protruding toward the right. The ring can be folded in the opposite way also to give a molecule looking just like the one represented except that the bond angles will be shifted so that the molecule faces left instead of right. When such a shift occurs, the hydrogen atom that was formerly in the equatorial position will

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protrude to the left, and the hydrogen atom that was formerly protruding to the right will now occupy an equatorial position. This shift can take place spontaneously.

Note the convenience of this shift for the occurrence of a coupled reaction between the 2 enzyme systems. The oxidized DPN may lie between the 2 enzyme molecules, rather like the filling of a sandwich. The triose comes in to pass a hydrogen atom to 1 side of the nicotinamide ring. Then the ring adopts the other configuration and passes the hydrogen atom on its other side to pyruvate. The cycle can be repeated indefinitely. We have achieved a coupled reaction without any necessity for separating the DPN from the surface of 1 enzyme and then combining it with another enzyme. Thus, the substrate of the lactic dehydrogenase is not free reduced DPN, but reduced DPN combined with the enzyme responsible for reducing the DPN in the first place.

In actual fact, the real evidence that the coupled reaction occurs without dissociation of DPN from protein was provided by kinetic measurements, first made by Cori, Velick, and Cori. They demonstrated that the coupled reaction occurs so rapidly that there isn't time for the DPN to leave 1 protein and then combine with the other. Their observations have been confirmed by Nygaard and Rutter. What I have been describing, with the help of models, is the reaction mechanism in terms of which the kinetic data can be understood. The point I wish to make is that even in the case of soluble enzymes, with freely dissociable "cofactor" substrates, the coupling between the 2 reactions may involve protein-protein contact. The physiological substrate of lactic dehydrogenase is probably not free reduced DPN, but reduced DPN bound to triosephosphate dehydrogenase. The analogy we have been seeking is thus found to be present. We have a hapten in the form of reduced DPN, bound to a protein—just the type of compound that might be expected to elicit a process analogous to specific antibody formation.

I don't believe analogical reasoning of this sort should be taken too seriously, but there is no harm in dreaming as long as we realize that we are dreaming.

Dr. Lwoff: In Dr. Gautheret's experiment is it clear that malignant crown-gall cells can synthesize auxins and that the normal cells are unable to do so? If I remember Dr. Gautheret's work, normal cells grown in the presence of auxin become independent of auxin for their growth and, at the same time, are malignant; so it seems clear that malignancy is bound to the possibility of synthesizing auxin. Is this true, Dr. Gautheret?

Dr. Gautheret: I think that the question is probably more complicated because we don't know whether it is an increase of auxin synthesis or a lowering of auxin destruction. There are these two possibilities, because we must also consider the question of multiplication of inhibiting substances. Perhaps the tumor transformation also concerns the balance of some inhibiting substances. So I must point out that, as Professor Street said, it is necessary to know the chemical constitution of auxin substances in the tissue. This begins to be known. What you said is perhaps true, but it is not yet proved.

Dr. Lwoff: Let us say that when a cell becomes independent of, and can grow without auxin, it is at the same time malignant. And in Dr. Woolley's experiments a malignant cell becomes able to synthesize vitamin B_{12}, whereas a normal cell does not. Dr. Thimann told us that the germinating spore was completely devoid of enzymes. This I am unable to believe because if the spore did not contain any enzyme how could it build up enzymes, whether in the presence of inducers or not? In order to synthesize something, enzymes have to be there. How does the germinating spore synthesize amino acids if it has no enzymes? In order to grow, it has to produce essential metabolites. If it does, this means that enzymes are present.

Dr. Thimann: Coenzymes are, of course, present. But no enzymes have yet been found.

Dr. Lwoff: Let us assume that the general principle of induction is correct. An enzyme is found in a cell in the absence of extrinsic inducer. If its synthesis is induced by an intrinsic inducer, then inhibitors of the synthesis of adaptive enzyme should also inhibit the synthesis of constitutive enzyme. This has been shown to be true in a certain number of cases. The synthesis of constitutive enzymes can be inhibited

by analogues of the inducer. This favors the hypothesis that an inducer is acting in the synthesis of constitutive enzymes. Otherwise how would an anti-inducer have an effect?

Dr. Potter: I would like to take up the cudgels here for Dr. Lwoff. I think that when experts disagree, the average person feels that he can relax again and wait another 5 years. This would be unfortunate. I think that the general concepts about ribonucleic acid and the enzyme-forming system of which Dr. Lwoff and others talked are valid. This story has been built up for a sufficient period of time so that one can say that in a general way it is fairly correct. We don't expect ever to understand the ultimate secrets of life, but these gentlemen are coming closer than was thought possible not so long ago. You are concerned in your association with the care and feeding of tissue. Now I would like to organize an association (and there are 1 or 2 members in the audience who might form the charter group with me) on the care and feeding of nucleoproteins. This is something that is in the foreseeable future. The remarks of Dr. Waymouth must be considered in the light of this afternoon's discussion. She raised the issue as to whether one should use a simple medium or a complex medium. I feel that the various additions you can make to the medium are enzyme inducers, anti-inducers, substrates, or antimetabolites (even though they are normal intermediaries, *cf.* Yates and Pardee: J. Biol. Chem. 221: 757, 1956). You are adding things into the middle of the metabolic stream. The cell fights its way out, if you give it enough time, but the cell is not dealing with the things that it is normally set up to deal with. What it does normally is to start with compounds that are at the beginning of the metabolic line (for the cell) and then feeds them into this system of induced enzymes that Dr. Lwoff speaks of, and sets up an equilibrium. When you begin to add compounds all through the metabolic pathway, the result is hopeless confusion. I think that one should attempt to analyze the media which the cells are normally confronted with and then try to work from that point.

Dr. Lwoff: I completely agree with Dr. Thimann that the permeases do not explain everything. When I told you about the memory of bacteria, I pointed out that when a galactoside is provided to bacteria devoid of permease, a few molecules are able to cross the bacterial membrane by diffusion, if the concentration is high enough. This explains why a number of substances can inhibit bacterial growth in the absence of a specific permease. Now when thienylalanine acts as an inhibitor of bacterial growth, it penetrates into the bacterium. This can be shown with labeled substances. When mutants resistant to thienylalanine are selected from a sensitive strain, it is found that thienylalanine does not penetrate into them. It seems as though, in this case, the resistance to the drug would be correlated with the loss of the specific permease.

Dr. Skoog: Probably, Dr. Lwoff, you have already said what can be said, but I was wondering whether you could define a bit more clearly the kinds and the nature of permeases? It seems to me from your discussion that the notion of such enzymes may have been introduced as a way out rather than as a way in.

Dr. Lwoff: The permease has, so far as we know, one function—to bring a given substrate from outside into the bacterium. Apparently the substrate is not changed by this transfer. It has not been possible yet to isolate permeases, and isolated permeases could not perform their function *in vitro*. What is known is that a bacterium can form β-galactosidase only in the presence of an extrinsic inducer. There is no permease in the absence of inducer. The permease is formed only when the inducer is added. Moreover, in order that the permease can be built, the bacterium must have the possibility to synthesize proteins; all the substances like 2,4-dinitrophenol, or azide, block the synthesis of the permease. Finally in order that the permease can bring the substrate inside the bacterium, energy is needed. If no energy source is present, then the substrate is not pumped into the bacterium. It is known also that the same gene controls inducibility *versus* constitutivity for β-galactosidase and for the permease. When a bacterium has a constitutive β-galactosidase, it has also a constitutive permease and *vice versa*. And it is very difficult to understand crypticity if one does not accept the permease hypothesis. In cryptic bacteria, enzymes are present

in the bacterium; they can be extracted and they are active. And yet the sugar, the lactose, or the galactoside, is not metabolized. It is not metabolized because it cannot penetrate into the bacterium; and it cannot penetrate because permease is missing.

Dr. Henderson: Concerning this form of habituation, which has been a complete puzzle to many of us since Dr. Gautheret discovered it many years ago, in the carrot tissue, one wonders whether it arises from a mutant cell, which takes on this characteristic growth, or whether it is a completely nutritive response in which the cell gets used to the medium. Thus, it seems to take the form of deadaptation rather than adaptation. It seems to me that in habituation we have always postulated a very delicate concentration situation as well as one of timing. It isn't a matter of 10 minutes, 1 hour or 2 hours, but a matter of weeks or even months, probably, depending on concentration. Can you, Dr. Lwoff, put this phenomenon of habituation in the same category with adaptive enzymes, inducers, inductive enzymes, based on the time schedule?

Dr. White: I suspect that most of the audience, not being specialists in plant tissue culture, may not understand what this word "habituation" means. It is a very specific term applied only to these plant tissues. It is the acquisition by a tissue during cultivation of the capacity to synthesize something such as auxin which it did not previously synthesize.

Dr. Lwoff: So far as I understand, it is not known whether the alteration is a mutation or not.

Dr. Henderson: So far as I know this has been eliminated. At least no one gives great credence to the mutation theory. It has been put on a metabolic basis. I am only thinking of the time and the concentration involved.

Dr. White: Since Dr. Morel has done a great deal of work with these habituations I suggest that we ask him for his comments.

Dr. Morel: I agree with Dr. Henderson. I do not think that this can be a mutation.

Dr. Lwoff: How has it been eliminated?

Dr. Gautheret: I think that it cannot be a true mutation, because the habituation, that is, the anergy, proceeds by steps. These steps may be large or may be small in any particular case. This phenomenon involves morphological changes which are very important, changes concerning the morphogenetic properties, and many other kinds of changes. It is not just a simple chemical change.

Dr. Lwoff: I do not think this would rule out mutation. The acquisition of resistance to penicillin in *Staphylococcus* is a stepwise process. A series of mutations controlling resistance to higher concentration of penicillin takes place, and these are accompanied by morphological changes: the original *Staphylococcus* which was gram-positive becomes gram-negative, and the mutant is able to synthesize a number of amino acids which it did not synthesize previously. So these mutations toward resistance to penicillin are somehow bound to mutations for the synthesis of amino acids and to what may be called morphological mutations. They are mutations, nevertheless.

Dr. Skoog: I don't think that habituation represents, strictly, the induction of auxin synthesis. Actually it is only a question of a quantitative difference between the normal and habituated strains. Presumably every piece of tissue may carry out some auxin synthesis. This can be shown very easily in stem cultures by cutting away the basal portions at successive intervals and observing the accumulation of material and growth, in time, at the basal ends of the remaining portions. For all we know, by actual tests, habituation may involve only the disappearance of an auxin-inhibiting substance. Perhaps it is more likely that it reflects an increased rate of synthesis of auxin and/or other growth factors, but there is no evidence at all that synthetic capacity in the qualitative sense has been acquired.

Dr. Lwoff: I would just like to take the opportunity to answer Miss Vennesland. If the substrate of any enzyme is necessarily carried to this enzyme by another enzyme, then you should never be able to obtain enzymatic activity *in vitro* by a mixture of a pure enzyme and of the substrate. It has to start with the pure substrate and the first enzyme.

Dr. Vennesland: I agree.

Dr. Lwoff: So it may be true for some but certainly not for all of them.

Dr. Vennesland: I did not intend to imply that this was a general phenomenon that I was discussing. You have to have a beginning.

Dr. Lwoff: Yes, and this hypothesis is very interesting because it explains why enzymes have to get bound together in a small particle and lie side by side. I think this hypothesis would account for many phenomena which we do not understand.

Dr. Henderson: I still wanted Dr. Lwoff to comment on the time factor in the formation of adaptive enzymes—induction of enzymes; the matter of a short time of induction *versus* a long time; during the latter time many things can happen. Isn't there a very definite time factor to which you must limit adaptive-enzyme formation?

Dr. Lwoff: I must confess I don't understand your question exactly. The inducer can be there for a very short period.

Dr. Henderson: Is there a time period beyond which you would say that adaptive-enzyme formation does not occur; that the adaptation must be due to something other than this very specific response?

Dr. Lwoff: No. For example, penicillin is added to bacteria and then destroyed with penicillinase. It is possible to show that the bacterium has absorbed and fixed a certain number of molecules of penicillin. Then the production of penicillinase is linear. The theory is that the penicillin is bound to the enzyme-forming system, which is, according to current hypotheses, a ribonucleic acid; then this template, which has been modified by the presence of the inducer, now produces the enzyme. Of course, when we speak of the inducer, we don't know at all in which state it is. It is possible that there is a metabolism of the inducer; that the inducer, when it combines with the hypothetical template, is not in the state of the original inducer as provided to the bacterium.

Senator Aiken: First, we are glad to have you folks holding your meeting here in Vermont. We are very happy we could give you some reasonably good weather.

I came into this hall while the last panel discussion was taking place and to tell the truth I didn't expect to understand a word that I heard spoken. But, as a matter of fact, I understood the very first word when the gentleman from North Carolina rose and said "crown galls," because at one time I was the largest producer of raspberries in New England and I used to observe and contend with crown gall in the production of that crop. And I wondered why it was that certain varieties of the same species would have crown gall and die; others would have it and survive; others would have it and kill off the crown gall. We would find the dead galls, and the plants would still go on living and producing for 10 years. Still other varieties were apparently absolutely immune. I used to wonder whether that went on with human beings as well as with raspberries. I suppose that you people will find the answer to that question sometime if you have not already done so.

I think your work is tremendously interesting. It so happens that for 2 years I was chairman of the Senate Subcommittee which observed the work of the National Science Foundation. And while they talked a language that sounded very much like Arabic to me, and I don't speak any Arabic, yet their work was so intriguing that even a layman could find it absolutely absorbing. I recall at one time, after they had been discussing matters, I made the observation that while they seemed to know all about heavy water, perpetual motion, and artificial satellites, I wished they would tell us how to detect adulteration of maple syrup. They said, of course, that would be easy, but I got a letter from George Merck, of the Merck Chemical Company, some 2 weeks later, saying that he had consulted all his chemists and there simply was no test to determine adulteration of maple syrup and there isn't to this day. That is just one of the little things that I was foiled on.

Once more I want to say how glad we are to have you with us in Vermont, and we hope that this weather you are enjoying today will continue during the rest of your stay. Thank you.

Dr. White: I can't resist the temptation, Senator Aiken, to take just a half minute to tell one story in regard to maple syrup. Wisconsin at one time wanted to compete

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with New England on maple syrup and being a dairy manufacturing community they thought they would speed things up by putting the maple syrup through the milk concentrator with which they made dried milk. That looked like an excellent idea, but when they got through, they had something that was perfectly white, which turned out to be nothing but cane sugar. In concentrating it, they had gotten rid of everything that characterizes maple syrup. So they had to abandon easy methods and go back to the old complicated one.

Probable Future Trends in the Nutrition of Tissue Cultures [1,2]

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Review of a set of detailed notes on the Hershey Conference of 10 years ago revealed that a number of key problems in the cultivation of cells *in vitro* were posed there. Equally impressive was the fact that since that Conference developments in animal tissue culture have been instrumental in bringing about an attack on some of these key problems, so that a new set of plans can be considered for the oncoming decade. To predict the future of anything, however—be it politics, economics, or the trends in the nutrition of tissue culture—is dangerous, and the last of these could well be the most dangerous. Nevertheless, an attempt to foresee probable lines of development in the immediate future may be most significant in advancing fundamental knowledge. It is to be hoped that a combined expression of opinion and a correlation of the problems and interests of everyone here can again bring into focus the key problems and possible approaches toward their ultimate solution.

The continuation of the introductory course on tissue culture given by the Tissue Culture Association, as well as the constantly increasing amount of graduate work that various institutions are making available, has advanced the whole field of cultivation of tissues *in vitro*. There should, of course, be continuing increase in this training with a close integration of it with cell physiology, biochemistry, and biophysics—particularly with respect to the practicalities now existent and the potentialities capable of development in the study of growth and development of tissues *in vitro*. Advancement of research and the sighting of new horizons require not only the development of improved instrumentation and methods but also trained and thoughtful intellects and leaders well integrated in tissue culture and the associated disciplines.

The increasing recognition of tissue culture as a scientific discipline has brought to general knowledge its usefulness as a weapon for the attack on many biological problems. All are familiar with the spectacular results from the use of tissue-culture methods in virology. This field alone has accounted for the growth of interest of a large number of organizations

[1] Received for publication May 28, 1957.

[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8–12, 1956.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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in the study of the growth of tissue cultures and their application on a scale unforeseen in 1946. Since the 1946 Hershey Conference, progress in basic facilities has made possible more accurate quantitative studies of the nutrition and metabolism of the cells. For example, with many cell types, cloned strains of cells have been made available. Also, plasma has been eliminated as a complicating substrate. These developments made it possible to prepare large numbers of replicate cultures routinely and accurately from cloned strains; more accurate and easily applied quantitative methods now are available for following changes in culture populations. It is also possible with such plasma-substrate-free cells to grow certain cell strains in relatively tremendous amounts.

Less than 25 years ago there could be found from the tissue-culture literature the concept that formulation of a completely chemically defined medium for the animal tissue cell was probably only an ideal, unattainable. At the present time, however, 2 cloned cell strains, 1 from the mouse and 1 from human epithelium, are being kept in a condition of relatively rapid proliferation in just such a chemically defined protein-free medium. The first of these, NCTC clone 929 [4] of strain L cells, is proliferating rapidly after more than 21 months. The second, NCTC clone 2271, a strain of skin epithelium from a 52-year-old man, has been proliferating in a protein-free medium for about 4 months.

These several developments represent some of the major basic current conditions, both applicational and experimental, from which may develop during the next decade the design of certain future trends in the field of study of the nutrition and metabolism of animal cells *in vitro*.

From an early date, studies on the nutrition of cells followed various general approaches in formulating media. There were numerous attempts to use extractives of naturally occurring materials, such as yeasts, tumors, and embryos, together with various sera. Even now, the majority of cultures are maintained on mixtures of such nutrient materials. Though more and more chemically defined materials are now being substituted, the field of research on these naturally produced extractives still remains an important one, particularly for the large-scale growth of tissue, since commercially cheap by-products in such industries as those producing antibiotics may ultimately offer cheap and easily handled sources of nutrients for animal tissue cells.

Almost from the beginning of research in the field of nutrition, analytical studies on the classical mixture of embryo extract and serum were carried out. The usual tissue-culture media of serum and embryo extract were fractionated by physical or chemical means, by various workers, to determine whether the medium could be simplified by omission of any fraction or fractions, with the idea that those that were still required might be analyzed further and found to be reproducible or that chemicals might be substituted for one or several of the fractions. Studies on the physical

[4] Since references to cell strains are becoming increasingly frequent in the tissue-culture literature, and in order to avoid confusion in the future, all strains originating in the Tissue Culture Section, Laboratory of Biology National Cancer Institute, will be designated by the prefix "NCTC strain" followed by the strain number Cloned strains will be indicated as "NCTC clone" followed by the strain number.

and chemical fractionation of embryo extract and horse serum have shown, comparatively recently, that the ultrafiltrate of embryo extract and the protein residue of serum were equivalent in growth-promoting ability to a mixture of unfractionated serum and embryo extract.

At the same time that analytical studies were being carried out, other groups of workers drew upon the knowledge available from nutrition studies on protozoa, microbes, and larger animals to endeavor, courageously, or perhaps rashly, to formulate chemical mixtures for growing mammalian tissue cells. These studies were of infinite value in stimulating research in the field and, in retrospect, the formulations themselves may have been less at fault than the sources of the chemicals or the methods and procedures by which they were evaluated.

Nevertheless, the newer knowledge of both nutritional, industrial, and analytical chemistry and technological advances in tissue culture in the late 1940's and early 1950's was required to make possible the formulation of the successful chemically defined protein-free media capable of maintaining continuous proliferation. Both Dr. Parker's group and the group at the National Cancer Institute have found that NCTC clone 929 of strain L can be cultured in a mixture of amino acids, deoxyribosides of nucleic acids, coenzymes, vitamins, reducing components, and a balanced physiologic saline containing glucose.

The protein-free chemically defined medium in use in the Tissue Culture Section, Laboratory of Biology, National Cancer Institute, contains many components. It is, nevertheless, comparatively easy to prepare, and is made up routinely in lots up to 20 liters; the components, of high purity, are readily obtainable commercially and cost about $9 a liter. NCTC strain 2071 of NCTC clone 929 cultures of strain L cells has been grown continuously in this medium, that is, with no breaks and in a state of reliable and rapid proliferation, for 21 months and through 56 transfer generations. In other words, it has been grown in a state of reasonably rapid proliferation completely free of any added or detectable extraneous protein for this period of time.

Other substrains isolated from NCTC clone 929 of strain L cells and subjected to this medium have also been tested to determine how easily they can be maintained on this chemically defined solution. With greater experience in handling cells in these solutions free of added protein, some 8 or 9 additional substrains of NCTC clone 929 of strain L cells have shown the same potential of rapid growth for indefinite periods of cultivation as NCTC strain 2071.

In order to test whether another cell type could be adapted to cultivation in this medium, cells of NCTC clone 2271, of human skin, were progressively depleted of serum in their nutrient medium through 3 transfer generations—a total of about 21 days. Since that time this cell strain also has been maintained, with relatively rapid proliferation, on this entirely protein-free solution for more than 11 transfer generations, or about 126 days.

Currently, no protein-free medium demonstrated to be capable of

indefinitely maintaining cell strains other than the two noted is reported in the literature. Other cell strains, tested for short intervals, as yet seem unyielding to continued cultivation. However, it is conceivable that by gradual alteration in composition of media, it may be possible ultimately to adapt cells of other strains so that they too may be cultivated in defined media, comparable to that used for the cell types that we have just mentioned. The nature of the type of cell adaptation required for the latter strains certainly justifies further detailed study.

The possibility exists that ultimately some medium similar to the one described, or some modification of it, will be found suitable for the rapid proliferation of a great variety of cell types and of even widely diverse cell types. It may be possible, through the use of a medium capable of maintaining proliferation of a wide variety of cell types to reduce greatly the amount of work required to determine the specific requirements for different cell types.

At the Hershey Conference, it was also pointed out that the bacteriologist devises media for specific purposes, and it has also been said that for a medium to support selectively the growth of 1 cell type only it must be exquisitely adjusted to that type. With a reasonably complex medium that is complete for many strains of cells it should become experimentally possible to define strain differences clearly by mechanisms similar to those used both by bacteriologists and animal nutritionists. The latter workers use a nutritionally depleted animal, and observe the effect of systematic readdition of dietary components on the test subject. This sort of approach requires, for complete appraisal, a base line of growth established by a diet free from undefined factors.

Furthermore, all fresh tissue, irrespective of origin, should be tested on one of the chemically defined media that has a wide variety of components and already known to maintain some cell strains successfully. This should be done to determine whether or not 1) their nutritional requirements are met, 2) the cells can be adapted to grow indefinitely, and 3) they can be made to function at least for the desired period of time. Up to the present such diverse fresh tissues as human pancreas, mouse spleen, mouse lung, and mouse connective tissue have been grown for from several weeks to a number of months on the chemically defined mixture.

As far as is known, no chemically defined medium free of protein supplementation will support any strain of cells indefinitely when the cultures are subjected to the relatively violent agitation now used with fluid-suspension cultures. Even where the nutritional requirements for a cell strain are met under a given set of cultural conditions, alterations in technological procedures or factors, such as this agitation during cell cultivation, greatly influence the possible successful use of any medium and may even cause its complete failure.

While cells of both NCTC clone 929 of strain L and the clone of human skin epithelium can be maintained in a state of rapid proliferation in this chemically defined medium, it should be clearly understood that the addition of 10 mg. percent of gross globulin or as little as ¼ of 1 percent

by volume of serum does further increase their rate of proliferation and also renders the cells less fragile. Therefore, one group of problems needing most critical attention, from both theoretical and practical points of view, is the establishment of the nature of, and the bringing under control and chemical definition, of the factor or factors associated with serum so definitely useful to the animal tissue cells so far studied.

With the addition of serum or serum protein, medium NCTC 109 has been found to substitute for embryo extract in the long-term cultivation of all the cell strains so far tested in our laboratory. Among these have been: 3 strains of mouse liver epithelium, 2 strains of HeLa, 4 strains of human skin epithelium, 1 strain of mouse mammary carcinoma, and 4 strains of mouse fibroblasts other than NCTC clone 929 of strain L.

To date, current knowledge indicates that any serum protein fraction tested will effect a change in the growth plateau. All protein fractions isolated from horse serum were found to promote growth of NCTC clone 929 of strain L cells when used as the source of protein supplementation. The degree to which these fractions could be substituted for horse serum without decreasing the optimal rate of cell proliferation appears, at this time, to be inversely proportional to the amount of chemical and physical manipulation necessary to separate and process the fractions. The globulin and albumin fractions, which have given the lowest increase in proliferation, were exposed during fractionation to potential denaturing influences to a greater degree than any of the other fractions. The gross globulin residue fractions, which could be substituted for the large molecular portion of horse serum or serum residue with no decrease or slight decrease, respectively, in rate of proliferation of the cultures, were not subjected to such severe treatment in the fractionation. These findings suggest that the growth-promoting component or components in horse-serum residue may be nonspecific protein, since all protein fractions tested possessed growth-promoting properties. In agreement with this concept, ovalbumin has been found to have some growth-promoting effect, but far less than that of any of the horse-serum fractions. Horse serum is either a much better source of protein than the egg, or the commercial ovalbumin preparation used was denatured or contaminated with traces of toxic or inhibitory material, such as fatty acids or higher alcohols. However, it is possible that a specific growth factor may be present, either a protein or some nonprotein component associated with the protein fraction. The evidence seems to suggest that if the factor were specific in its action, the effective component would have to be present in all other fractions tested and present in some in very minute amounts.

Another possibility is that the protein may provide the cells with an additional amount of an agent already in the chemically defined medium, but in growth-limiting amounts. One other possibility is that this protein may have a protective effect against a toxic agent—that is, the sulfhydryl groups may combine with traces of heavy metals, which could be inhibitory to growth. Solution of this problem—to determine the function of serum residue, or fractions thereof, in influencing increase in growth—might well

suggest formulations for other chemically defined media for strains of cells not yet tractable to growth on the protein-free chemically defined media now available.

The problems associated with obtaining more massive growth and for culturing fastidious cell strains therefore seem to resolve themselves, at least for the moment, into obtaining well-characterized fractions of protein that can be produced relatively cheaply and of such quality that cells will grow well and in adequate amounts for use in the study of innumerable problems. In addition, as knowledge advances, the possibility of substituting the seemingly essential protein fractions must be explored.

If the amount of growth obtained with embryo extract and serum is to be the reference standard, then the means of obtaining this amount in a single culture flask, in a given time, is a problem that still has to be solved for cells on chemically defined media.

But despite this, there is an entire spectrum of cell types that have not produced strains suitable for study when cultivated in classical stock media. In the next decade these will offer the greatest challenge for nutritional studies. Among them are cells recognized as having highly specialized functions, for example, those with highly specialized secretory roles. Such cells have not yielded to satisfactory long-term cultivation on media containing either natural components or completely chemically defined constituents, which forcibly points up the fact that, though there are cells that can be maintained in a state of rapid proliferation in chemically defined media, only a beginning has been made on an even greater problem. Control of the nutrition of the cell to promote proliferation is an important aspect, but achieving nutritional control for this purpose alone is not enough. Control of the cell's nutrition, as this nutritional control may influence specific differentiation, function, and metabolism, must still be achieved. Enzyme studies may serve in a more definitive way to determine the requirements of such highly specialized cells, and it is only through study of the total metabolic process of pure cell strains that dynamic requirements may ultimately be learned and put to practical use. Studies with the electron microscope and the use of tagged substances may aid materially.

Though large amounts of nutritional and metabolic data on cells have been collected from short-term cultures with mixed-cell populations on ill-defined or semidefined media, all such data, if the objective justifies it, must ultimately be confirmed with completely chemically defined media on pure strains in long-term stabilized cultures; our information may or may not be revised, but this cannot be forecast. Furthermore, there is almost no problem that has been studied with tissue slices that, probably, would not be more satisfactorily and more conclusively solved with pure strains of cells *in vitro* in chemically defined media. Under such rigidly defined conditions, it would be possible to study more effectively differences in nutrition and metabolism of cells uninfluenced by the mixture of cells found in tissue slices and by their interdependence. Such studies

can be used in the solution of problems of growth of both the neoplastic cell and the normal cell from which it arises.

It appears that cancer must be a metabolic disease, in the sense that it is a disturbance between the interrelated metabolism of the malignant cells and the cells of the host. This will probably be one of the most significant areas of study during the next decade. A clearer understanding of the metabolism of the malignant cell and the normal cell from which it arises, as well as an understanding of the transition of the normal to the malignant cell, must ultimately lead to a better understanding of cancer in the laboratory and as an almost inevitable consequence, in the clinic.

Tissue for reparative surgery also is badly needed. Cultivation *in vitro* of functioning endocrine tissue for implantation deserves concentrated research effort in the near future. The embryologist and endocrinologist need to achieve an understanding of the interrelationship and interaction of the nutritional requirements of cells. These offer great fields of study. From pure strains of cells, the physiologist may ultimately build organs, not necessarily characteristic morphologically but capable of physiological function.

Characterization and classification of cells by the fastidious definition of their nutritional requirements is still another critical area for research effort. This almost surely must extend beyond simply characterizing them as fibroblasts, epithelial cells, and wandering cells; ultimately the research efforts may characterize and classify cells by indices of the niceness of their requirements for specific nutrients, and by the niceness of what they produce from these nutrients. Such refinements of classification have already been achieved with bacteria. It is hoped, and certainly the expectation is great, that such niceness of requirements may be carried to the point that differentiation can be controlled and a biochemical correlation can be made with this controlled differentiation.

Many, if not most, of the advances in animal nutrition have been strongly motivated by sociologic and economic demand. Tissue-culture nutrition has now reached the stage of development where these same strong forces are able to and will influence future trends in nutrition of animal cells *in vitro*.

DISCUSSION

Dr. Morgan: Predictions of future trends, or probable future trends, in tissue-culture nutrition are inevitably colored by the personal interests and previous training of the predictor. Within those limitations, I would like to suggest certain possible future trends and also include some data which, I think, will supplement the paper that Dr. Evans has presented. After the earlier discussions, it seems to me that there is really only one safe prediction that can be made. That is, since the past 10 years have been extremely productive in all fields of tissue culture, the next 10 years should be even more so.

From the discussions of the previous panel there does appear to be a similarity between the present status of tissue culture and the status of such allied and collateral fields as bacteriology about 25 years ago. From those early beginnings we have witnessed the development of synthetic media for many types of bacteria and of such new fields as bacterial physiology. It seems a reasonable prediction that future studies with tissue culture may follow much the same pattern. By cultivating tissues *in vitro* free from the complex interconversions that, in the whole animal, occur in such organs as the liver, we may be able to discover the final form in which different cells actually utilize their nutritive substances. In other words, we may be able to develop a comparative biochemistry of cell types.

From recent studies on tissue-culture nutrition we can make a start on such a comparative biochemistry. Table 1 summarizes some of our present knowledge of the amino acid requirements of different types of cultures as compared with the requirements of the whole animal. The tissue-culture requirements are shown in the center columns. When compared, the patterns are remarkably similar. In particular, the essential amino acids for these different types of cultures appear to be almost identical. There are occasional differences, such as the requirement of serine by rabbit fibroblasts

TABLE 1.—*Amino acid requirements of various types of tissue cultures, of the chick, and of man**

Amino acid	Chick	Chick-heart fibroblasts	Strain L	HeLa	Rabbit fibroblasts	Man
Arginine	+	+	+	+	+	−
Histidine	+	+	+	+	+	−
Lysine	+	+	+	+	+	+
Tryptophan	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+
Tyrosine	−	+	+	+	+	−
Cystine	−	+	+	+	+	−
Methionine	+	+	+	+	+	+
Serine	−	−	−	−	+	−
Threonine	+	+	+	+	+	+
Leucine	+	+	+	+	+	+
Isoleucine	+	−	+	+	+	+
Valine	+	+	+	+	+	+
Glutamic acid	−	x	−	−	−	−
Aspartic acid	−	x	−	−	−	−
α-Alanine	−	x	−	−	−	−
Proline	−	x	−	−	−	−
Hydroxyproline	−	x	−	−	−	−
Glycine	+	−	−	−	−	−
Glutamine		−	+		+	

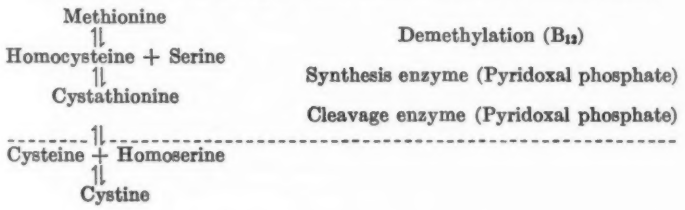
*+=Essential; −=nonessential; x=inhibitory.

and the nonrequirement of isoleucine by chick embryonic-heart cultures, but these are isolated cases. They may be related to the specific tissues, and they certainly need further investigation. When these culture requirements are compared with those of certain whole animals, there is again general similarity but also two major points of difference. All the tissue cultures studied so far require both phenylalanine and tyrosine and also require both methionine and cystine. In the whole animal this is not the case. The reasons for this difference require further investigation.

Although considerable effort has been expended on the study of tissue cell nutrition, the field of tissue-culture metabolism has been virtually untouched. With synthetic media available at the present time, even though admittedly those media are imperfect, it is now possible to study the effect of the medium upon the cells and, concurrently, the effect of the cells upon the medium. We have studied the changes in amino acid content of our synthetic medium during cultivation of chick embryonic-heart cultures and have demonstrated patterns of amino acid uptake and accumulation. These patterns can, in turn, be correlated with the amino acid requirements for survival of those cultures: The essential amino acids disappear from the medium and the amino acids that are either inhibitory or nonessential accumulate. With other types of tissue cultures we find different patterns and, in the future, we hope to employ this correlative approach to research on nutrition and metabolism in attempting to develop synthetic media specific for other cell types. As a prediction, I think it might be safe to say that this type of correlated research offers considerable promise for the future.

Because of the essential role of the sulfur amino acids in tissue-culture nutrition we have studied them in some detail. We found that chick embryonic-heart cultures in synthetic media have an absolute requirement for L-cystine and a supplementary requirement for methionine, which can be satisfied equally by either the L or D isomer. When our sulfur studies are compared with those concerned with the pathways of sulfur metabolism in the whole animal, as shown in chart 1, it appears that in the cultures there may be a metabolic block in the sequence of interconversions leading from methionine to cystine. From our studies so far, which are not complete, that area of possible metabolic block appears to lie in the cystathionine region, as indicated by the dotted line in the chart. We are studying these reactions at the moment and are, in fact, trying to carry out enzyme and coenzyme analyses of the cultures for this specific system. We also have begun tracer studies to follow the precise steps in the metabolic pathway. Information of this sort on precise metabolic pathways is very badly needed in tissue culture. It is a basic requirement for future progress, I think, particularly in looking for differences between normal and malignant cells of different types. This chart is shown not so much to indicate results actually accomplished as to suggest that even though our culture systems and synthetic media are still imperfect we can do precise metabolic studies.

CHART 1.—*Pathway of sulfur amino acid metabolism in animal tissues*



At this point, I meant to bring up the question of cell types as opposed to fresh tissue and the question of the mutation and variation in all the established cell lines. That point has been so thoroughly discussed in previous sections that I hesitate to start the argument over again. Instead, I would merely point out that this factor must be considered at all times.

Dr. Evans mentioned the advantages of nutritional-depletion techniques in bacteriology and animal studies. We have worked out such a nutritional-depletion technique and find that, by using it, we intensify the coenzyme A requirement of our cultures approximately 2,000-fold, apparently without altering the metabolic pattern of our cells. This type of work makes it possible to determine nutritional requirements in the future with a greater degree of precision than has been possible in the past.

In conclusion, I would like to come back to what I think is essentially my main prediction for the future. Although a start has been made on tissue-culture nutrition and a beginning made on nutrition in its relation to metabolism, I believe there are still great areas of complete ignorance. We know very little about the energy requirements of our cultures in relation to all the processes that we are studying. We don't know very much about the precise metabolism of any nutritional factor. In the future, I think there will be an increasing trend in that direction and, I believe, precise studies of that nature will provide the key that we hope will unlock many associated problems.

Dr. Boll: My approach to this question will not be either to discuss problems in which I am specifically interested or to review all the problems which have been or will be described at this meeting—problems such as trace-element nutrition, the use of solid media, carbohydrate requirement, and so on. Each of these problems is interesting in itself and often of special interest to a particular worker. But the interests of individual workers do not constitute a trend. It might, perhaps, be more politic to approach the question of probable future trends by asking: What are the future trends in plant physiology and will the problem of tissue-culture nutrition be of importance in those trends? This is too big a topic to discuss in the time available but a few possibilities can be mentioned.

The general problem of plant-growth regulators continues to be the center of interest in plant physiology. The detection of substances that affect only cell expansion does not require the use of tissue culture. It is for the study of growth as a product of cell division and cell expansion that tissue culture has proved especially valuable. We now have a number of tissue cultures that are being used, or could be used, for the routine testing of plant extracts, liquid endosperm, and other materials suspected of having biological activity. Two aspects of this type of work should be mentioned. First, the continued attempts to characterize the substances that, together, constitute the growth-promoting activity of coconut milk and, second, the discovery of the biological activity of kinetin.

Some of the active components in coconut milk have been characterized, but the identity of other components is still obscure. Furthermore, we are now aware of other examples of liquid endosperm in plants (*e.g.*, horse chestnut and certain grasses). Dr. Gautheret stated that there are at least 15 examples. Others will probably be found. Each of these must be considered as a possible source of nutrients of value in plant tissue culture. Conversely, plant tissue culture is the tool whereby the activity of an individual substance, in the endosperm, is detected.

Increasing attention should be paid to the testing of endosperm of a variety of plants and to the use, as bioassay systems, of a number of different tissue cultures each of which shows some response to a liquid endosperm or component thereof. There are a number of plant tissue cultures that can be maintained on complex media containing either coconut milk, yeast extract, banana diffusate, or other plant extracts. Each one of these cultures is a potential bioassay system. It is interesting to note, in considering Dr. Evans' paper, that whereas students of animal tissue culture are searching for synthetic media, the students of plant physiology are searching for sources of complex, undefined media from which growth regulators may be isolated.

Since the discovery that kinetin promotes cell division in cultures of tobacco tissue, it has been shown to act as a growth regulator in a number of plant growth systems. Furthermore, its activity is reproduced by a number of structural analogues. The significance of kinetin and its analogues in dedifferentiation, and the initiation or stimulation of plant tissue cultures in general, remain to be evaluated.

Associated with the interest of plant physiologists in growth regulators is an increasing interest in specific problems of morphogenesis and differentiation. Auxins appear to be universal regulators of such morphogenetic processes. The mechanism of their action is still an enigma, the solution of which could come from studies of the effect of nutritional conditions upon morphogenesis rather than upon cell expansion alone. Skoog's studies on the effects of nutritional treatments on the morphogenesis of buds and roots in tissue cultures have already contributed to current hypotheses concerning a relation between auxin and the transfer of high-energy phosphate.

The solution of many problems in plant physiology and morphogenesis would be brought nearer if we could obtain free cell cultures from tissue cultures or directly from plant tissues. The nutritional problem is, therefore, to find a medium which will induce the growth of free cells. An important step in this direction has been made recently in Dr. White's laboratory, where tissue cultures have been converted to more or less free cell cultures. The substances that bring about this change are known substances. Thus we have a clue to the metabolic systems that control the separation of the cells. We can expect that these results, and others mentioned in this symposium, will stimulate further work on this problem.

Dr. Bonting: I would like to make a brief remark concerning an area where, possibly, more work should be done. We have heard much about changes that take place, both morphologically and with regard to nutritional requirements, in cells in tissue culture when they are grown for a long period of time and are subcultured several times or when they are established as a single cell strain. It would seem to me that this raises the question of what significance our findings in tissue culture have for the *in vivo* situation. I believe that for most of us the stimulus to do tissue-culture work is to obtain information about the *in vivo* situation, or at least to deepen our understanding of the latter. Such changes of morphology and nutritional requirements might also be a matter of interest for the study of cell differentiation and dedifferentiation.

In addition to the many instances of such changes that have been cited by previous speakers, I might perhaps give 2 or 3 examples. Dr. Morgan and his associates (*1*) recently found different patterns of amino acid metabolism in terms of amounts of amino acids taken up or excreted, depending on the length of cultivation. Dr. Swim and Dr. Parker (*2*) found a difference in morphology for an established cell strain (U12) of human uterine fibroblasts and for cultures of the same tissue that were less than 4 to 8 months old. More recently, Dr. Swim (*3*) established several lines of this strain U12 that differ in their response to various media and still others that differ markedly in their requirements for vitamins. And finally in some experiments carried out by Marion Jones, Robert Featherstone, and myself (*4*), we found a decrease of cholinesterase activity in chick-embryo intestine on cultivation. This decrease could be abolished by addition of acetylcholine to the medium.

On the other hand, there are also indications that cells *in vitro* can maintain their functions unchanged for a remarkably long time. Recent work by Dr. Crain (*5*) showed that little change occurred in the action potentials of chick-embryo spinal-ganglion cells cultivated *in vitro* for 7 weeks. It seems to me that more systematic study is needed in this area. Perhaps the best approach would be to determine the activity of a wide variety of enzymes for the original tissue, the explanted tissue grown for varied lengths of time, and where possible, for strains obtained from this tissue. This whole matter of changes between the *in vivo* situation and the tissue-culture situation also adds another factor to our discussion this morning—that of simple *versus* complex media. If these changes are adaptations of the tissue to a new environment, then the use of simple media might draw us away rather than bring us closer to the *in vivo* situation. It would, therefore, be well to consider this factor when choosing a medium.

Any work done on this subject might enable us in time to come, perhaps at the next decennial review conference, to defend ourselves against Dr. Skoog's remark of this morning that tissue-culture nutrition studies have no significance for the understanding of the nutrition of the whole organism.

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(*4*) Jones, M., Featherstone, R. M., and Bonting, S. L.: The effect of acetylcholine on the cholinesterases of chick embryo intestine cultured *in vitro*. J. Pharmacol. & Exper. Therap. 116: 114–118, 1956.

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Dr. Hull: Not having done any basic research in cell nutrition, I feel quite out of place on the program today. Perhaps the only reason for my being here is to say what I can on the practical applications of tissue culture and how media and their constituents influence these practical applications. I would like to repeat two comments that have been made. One concerns the adaptation of certain lines of cells to growth on synthetic medium. I would like to point out that in the few instances where cells have been grown for long periods of time, through many generations, on a purely synthetic medium, specially adapted cell lines were employed. Such growth cannot be obtained with all or with many cells of the strains now available. One wonders what alterations have been made in the adapted cells. If one is interested in virology, it is important to know whether their sensitivity has been changed. If one is interested in some other phase, such as malignancy, perhaps their ability to produce tumors has been changed. Even though we, who make practical applications of tissue culture, are indeed grateful for all the work that has been done by the various speakers, we still realize that there is a lot more to be accomplished before we can use synthetic media widely on all cell strains with which we would like to work.

Several people have also mentioned differences in the type or quantity of growth of cells in various media, in stationary cultures, and in fluid cultures. We likewise have observed these differences and find that certain cells, which grew very well on a synthetic medium or nearly synthetic medium in conventional flasks, would not grow at all in fluid cultures with the same medium. This may be entirely physical rather than nutritional. One difference may be aerobic *versus* anaerobic metabolism. Certainly fluid cultures, such as those used in the National Cancer Institute and in our laboratory, were more aerobic than were the conventional flasks. Whether this suggestion is valid and whether it influences the nutrition of cells in these two cultures, I do not know. Again, speaking from the practical standpoint, through the past 10 years the various workers in this field have formulated media which are very close to the type for which we are all searching. But we see a huge list of highly purified chemicals, enzyme substances, coenzymes—materials which are very expensive and which are often hard to obtain. Once the nutritional requirements of cells have been worked out, it will be up to us to go back again and try to find cheaper sources of all these materials. This has been true in bacteriology and it will be true again in tissue culture. When we work with hundreds of liters we cannot use purified coenzyme A or some of the amino acids. Also, I would be pleased to see more effort made to reduce the number of ingredients. I think Dr. Eagle has done some very nice work in this direction. I have always felt that there are many substances, *e.g.*, in medium 199, which are not necessary.

Another problem that faces us in using tissue cultures or tissue-culture fluids in human therapy is the problem of what the human or the animal can tolerate. Obviously the old tissue-culture media containing serums, extracts, and plasma cannot be inoculated into human beings. Currently we are using medium 199, in large quantity, and over 100 million cc. have been inoculated into people in the last 3 years. Apparently it has produced no problems. I have handed the formula of Dr. Evans'

medium NCTC 109 to a biochemist, a pathologist, and a pharmacologist. All 3 have assured me that there is nothing in this medium which could not be tolerated by human beings. We are also concerned, especially in virology, with the sensitivity of our cultures. When one is forced to use serum, even in small quantities, the sensitivity of cells to virus is greatly reduced. This loss of sensitivity brought about by low serum concentration can be eliminated, however, by passing the cells for 1 generation without horse serum or calf serum. However, if one uses large quantities of serum, such as 20 or 30 percent, we have found that the inhibition produced by the serum cannot be rinsed away; in fact, the cells have to be grown away from it for at least 3 generations. It has also been reported by several people that omission of certain ingredients from medium 199, or addition of others, has influenced cell sensitivity; so we must guard against or check this possibility at all times.

Other factors that influenced media very directly are filtration and inactivation of viruses by formalin, *gamma* ray, or other forms of irradiation. We have recently been carrying on some work with another laboratory in which we have been attempting to inactivate virus with *gamma* irradiation. If one treats virus in medium 199 with sufficient roentgens to kill the virus, the medium is converted into a highly toxic mixture. Also, media can very directly influence tests that one may wish to make on drugs. Elsewhere, we have reported studies with an antibiotic material that will protect cells from certain viruses, and we have described our studies on the effect of medium on this protection.

I hope that Dr. Morgan, Dr. Evans, and others who have touched on the subject of the biochemical and physiological properties of cells as a means of identification will continue this work so that in the future we may be able to use methods of this type for identifying the many cell strains that we have in our laboratories.

Dr. Harris: I would like to comment on the use of the word "adaptation," as Dr. Evans used it in relation to protein-free media and as Dr. Hull has employed it. It is used commonly in tissue-culture discussions. There is a very basic ambiguity in this word that I do not believe has been sufficiently appreciated in our thinking, although the microbiologists have faced this problem long ago. In microbiology the word "adaptation" has been used in several ways. It sometimes covers enyzmatic adaptation, or induced biosynthesis as it is better known nowadays. This is a phenotypic change that can be reversed when the inducing conditions are removed, leading to loss of the induced enzyme. In general, this is a change involving the entire population. We also speak of adaptation as the result of mutation and selection. Certainly this is a favorable change, so that the population is "adapted" in a sense, yet by an entirely different mechanism than the phenotypic change represented in the enzymatic adaptation. Perhaps this distinction is not altogether academic in relation to our concepts of cell strain, for when we say that they are "adapted," meaning a physiological or phenotypic adaptation, presumably this would connote something similar to the idea of modulation as Weiss and others have used it. It would mean that 2 strains, one of which was adapted and the other was not, would not differ except superficially in their physiological adjustment to the environment. If, however, from this use of "adaptation" we infer a genetic change, then if one cell strain is adapted and the other is not, we have 2 genetically distinct cell types that may have fundamental and inherent differences. To bring this matter into our discussion of nutrition and metabolism, if we have cells that are adapted, for example, to protein-free media and this represents a genetic change, then the nutritional data that we obtain pertains specifically to this strain and we can transfer the findings only with difficulty to nutritional characteristics of other strains. If the phenomenon is a physiological or phenotypic change, we may have a much greater latitude in our interpretation of its significance for other cells. Through the use of clones and appropriate statistical methods it should be possible to clarify the nature of these "adaptations" as we describe them. It would seem that one of the most important future trends in nutrition will be the resolution, in specific instances, of what this ambiguous term "adaptation" really means.

Dr. Evans: I said that I thought one of the future problems will be to learn what happens when we get several substrains of cells from a given cell strain accustomed to growing on a chemically defined medium. I don't think we have attempted here or in the literature to use the word "adaptation" in much of any sense except that of getting cells accustomed to growing on the medium that we give them, just as when we start with fresh explants on naturally occurring medium we very frequently have to get them accustomed to growing on that also. That is all we mean by the word "adaptation" at this stage of the game.

Dr. Raper: I may be the only one here that needs this clarification, but I would like to hear a little clearer definition of a clone and of a substrain. What do we mean by a clone and what do we mean by a substrain?

Dr. Evans: A clone strain of cells, in our laboratory, is one which has been grown to full culture size and then to many cultures from a single cell. A substrain is simply, in the case of this little discussion here, the same cell type carried on a different medium. The strain designated NCTC 2071, is merely the clone strain of strain L carried on a chemically defined medium for almost 2 years. It is a bit of an arbitrary designation in order for us to keep our cultures thoroughly separated and catalogued in our own laboratory so that we won't have them first on chemically defined medium and then on stock medium. It is as much a matter of cataloguing as anything else.

Dr. Woolley: Dr. Evans mentioned the requirement for protein in the culture of animal cells. Some of the things she mentioned made me think about a factor of bacterial growth called strepogenin that has been worked on for many years. This growth factor is obtained as a peptide by digestion of the plasma proteins. It is found only in traces in egg white, which would fit with her observations on the relative potency of sources of the substances required for animal cells. This substance has been isolated in crystalline form and synthesized. I think that in the future we may see attempts to determine whether the protein requirement of animal cells can be replaced by this peptide.

Dr. Morgan: On the question of strepogenin, we did add it to our synthetic medium. We tried it several years ago and didn't see any major effect on either survival of the cultures or increased protein formation. However, I should emphasize that we didn't use the crystalline strepogenin but rather the crude source, liver fraction L. These experiments should be repeated with the purified material.

Dr. Evans: I think it needs to be used only under those conditions where we cannot grow cells without such a protein or protein derivative, or protein fraction or substitute; for example, it can be used in those conditions where we find the cells apparently altered or weakened or we may even find the membrane more sensitive in its absence. Maybe it has a physical effect rather than an actual growth-promoting or nutritional effect.

Dr. Earle: I want to say a word about the term "adaptation." As we try to describe what we see and observe, we find that various specialized fields have taken over many general words and are now using them almost with a proprietary label, in special senses. As we have used the term "adaptation" it carries no implication of mechanism. This lack of implication is very deliberate on our part. We have seen gradual changes in cells that made them more rapidly proliferative in defined culture media. We have also seen sudden changes in cells. Whether these changes arise from genic changes in certain instances, and whether they arise in other instances from changes in the mitochondria or Golgi or other cytoplasmic organs or in an enzyme system, is not implied, as we have used the term "adaptation." We have deliberately tried to use as general a term, absolutely free from implication of mechanism, as we were able.

I am sure the term "adaptation" has been used in specialized senses in various scientific areas. But we are reaching a little of the state of frustration when we try to find a word that has not been used in a special sense. We are beginning to wish that somebody would put up a few fences and say, "In this area we will try to keep words that will not be used in special senses of implication of mechanism." This use of the general term has been made deliberately. In our experience we do not know the

mechanism of these changes; we are extremely suspicious that they are not of uniform origin; some of them are gradual, some of them are sudden.

In looking back over the history of tissue culture, it is my impression that one of the errors that has been made repeatedly in the past is the use of overly specific terms that carried a definite implication of mechanism before adequate data indicated clearly what the mechanism was. In other instances, a general term has been interpreted by a commentator as implying an overly specific mechanism. I would consider these grave dangers in the use of words should be most carefully avoided.

Dr. Evans: We have not made this sort of test with the HeLa cells. I do know, however, that in our laboratory we have a strain of HeLa cells that has been adapted to grow for quite a long time now on horse serum instead of human serum. This is a little piece of work that we did in collaboration with our colleagues across the street at the Navy. I suspect, therefore, that we might find with this particular strain of cells that the *horse-serum fractions* might work just as well as human serum does. I don't want to create the idea that I am in favor of the use or the addition of protein fractions to any chemically defined medium. I think that I repeated at least 3 times, perhaps 4 or 5, that this medium with which we have had pretty good success for almost 2 years has no protein in it—no detectable protein is present, nor has any extraneous protein been added. It is only if we wish to use it with other cell strains not yet tractable to growth on protein-free medium that we find that we need to continue studies determining the function of protein, but we are not making any plans to continue to use proteins in our media beyond trying to determine what the function of protein is.

Dr. White: May I make one comment in regard to HeLa cells. They grow perfectly well on rabbit serum. And the routine concentration that we use is 5 percent, which is considerably below that usually used with human serum. There is no adaptation period required. You can go direct from one type of serum to another. I think that in part answers the questions. I suspect we will get rid of the serums entirely very soon.

Dr. Potter: I think the question of the blood is a very interesting one, following Dr. Waymouth's discussion of the fact that the blood has things that are thrown out as waste products by the various cells. It might be of interest to get blood as it comes from the kidney, when it might be purified somewhat, and then to use this blood by separating it into the acid-soluble and the acid-insoluble fractions. There are methods available for separating all the nucleotides, and these methods are quite different than they were a few years ago. When we study a sample of liver we now very frequently separate it into 2,000 fractions, using gradient elution. There are a great many nucleotides that are as yet unidentified. I believe it would be possible to do the same thing with blood. I am aware that people have studied the protein fractions a great deal, but I think some of the protein fractions might be thought of as being growth inhibitory. You might ask the question, "What do you have to take away from blood in order for it to permit growth in certain tissues?"

Histogenesis in Plant Tissue Cultures [1, 2]

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Plant tissue culture, in its current orientation toward physiology and biochemistry, has tended to abandon its original domain of histogenesis. In some cases investigators seem even to forget that their cultures are made up of cells. It is probably for this reason that Dr. White has asked me to recall to you the sequence of events in the development of tissue colonies and the principal structural characteristics that result from those events.

There are 3 basic types of proliferation—that of unorganized parenchyma, that of specialized cambial layers, and that of organized growing points. We will examine these 3 types of proliferation, first as they occur in initial explants and then as we find them in established colonies.

Let us first consider initial explants. A fragment of carrot phloem may produce at its surface a thin layer of new growth (fig. 1, plate 40). This results from a generalized multiplication of all its surface cells. In other explants only certain cells scattered at random over the surface multiply, so that the culture becomes covered with discrete "pseudothalli," or the interior of the explant may proliferate in a diffuse fashion, giving rise to a general thickening of the mass. This occurs in cultures of lilac stem or of the rhizomes of *Amorphophallus*, as shown in text-figure 1. In other cases, vessels and sieve tubes as well as excretory organelles, such as the secretory and laticiferous canals, are accompanied by parenchymatous cells that have a higher proliferative capacity than do parenchymal cells in general. The same is true of the parenchyma of the medullary rays. As a result of the activity of these localized parenchymal meristems, cultures of certain plant materials may become covered with isolated pustules of new growth. The perivascular cells of the radish and the phloem companion cells of the carrot represent examples (figs. 2 and 3, plate 40). Parenchymal cells are poor in cytoplasm (text-fig. 2*A*). The nuclei are small, the plastids are rich in storage materials, and there are large vacuoles. Parenchymal cells that accompany conducting or secreting elements have the same general structure but are less rich in paraplasmatic materials, as Buvat has shown for the latex tubes of chicory (text-fig. 2*B*). Their plastids do not elaborate starch or other

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[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8-12, 1956.

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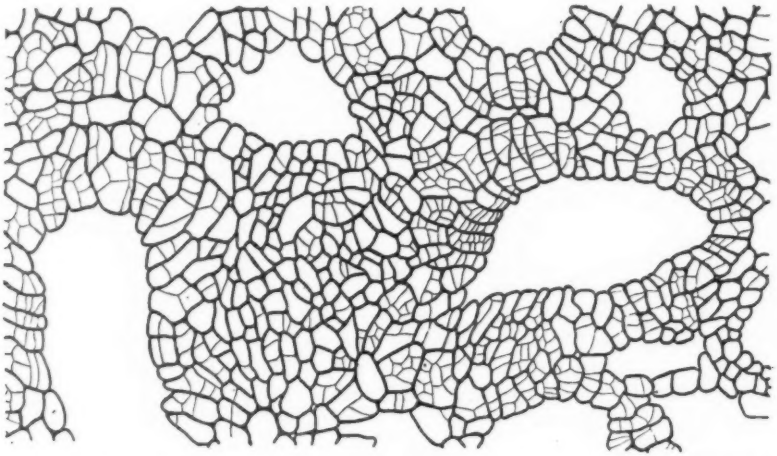
products. Their degree of differentiation is thus intermediate between that of common parenchymal cells and cambium, which we will examine later. This explains their particular tendency to multiply.

Now let us consider cambial proliferation and differentiation of fibrovascular structures. If one isolates a fragment of an organ that contains an already organized cambium, the latter will proliferate actively and produce an important mass of parenchyma containing considerable quantities of scattered vascular bundles that consist of phloem associated with xylem.

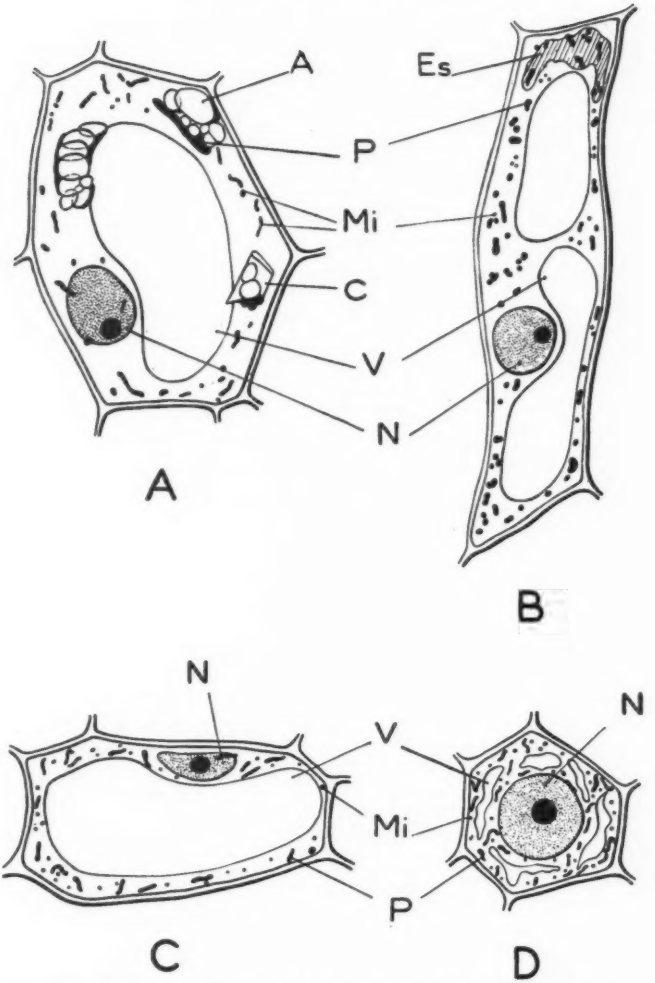
Cambiums may also form *in vitro* by dedifferentiation and reorganization of parenchymatous elements. Once formed they will then proliferate quite like pre-existent cambia. Such newly formed cambia may



TEXT-FIGURE 1.—Section of a bit of tuber of *Amorphophallus rivieri* cultivated for 3 weeks in a nutrient containing coconut milk. The originally parenchymatous mass of the explant has multiplied in a completely anarchic manner.

be well or poorly defined. Sometimes they are diffuse. This is generally true if a growing tissue is placed in contact with an aqueous medium. If, for example, a fragment of xylem of Jerusalem artichoke is treated with an aqueous auxin-containing nutrient, the region of the explant that is above the nutrient surface produces a parenchyma without organization, while the submerged portion proliferates in an orderly, regular fashion. The cells below the nutrient surface divide perpendicularly to the direction from which the auxin penetrates so that the newly formed cells are arranged in regular lines (fig. 4, plate 40). Cytologically, in matters of nuclear size, vacuoles, etc., this newly formed tissue is similar to the parenchymas.

In other cases, the cambia are better defined; some form cork cambia, others, vascular cambia. Cork cambia are formed at the surface of cultures of chicory, *Scorzonera*, etc., between the lignified surface cells and the internal parenchyma.



TEXT-FIGURE 2.—Principal types of cells that are capable of proliferating.

A. Parenchyma of which the parenchymatous cells of carrot phloem are typical. The nucleus (*N*) is small, and the plastids (*P*) are filled with starch (*A*) and crystals of carotene (*C*). *Note* the large vacuole (*V*), (*Mi*) mitochondria (after Buvat).

B. Secretory cell, a lacticiferous cell of chicory. The general structure is similar to that of parenchyma but the plastids contain neither starch nor carotene. This type of cell is less differentiated than is parenchyma (after Buvat)—(*Es.*) oleoresin.

C. Cambial cell. Like the parenchymal cell, it has a large vacuole but the plastids are undifferentiated and produce no starch.

D. Cell of a vegetative growing point (primary meristem). These cells are small, rich in cytoplasm, with relatively large nuclei, small and diffuse vacuoles, in the form of filaments or distinct droplets, and with undifferentiated plastids devoid of starch.

Fibrovascular cambia may also appear at the boundary between 2 other tissues. If, for example, one isolates a piece of xylem of carrot, the surface cells will proliferate to give a homogeneous parenchyma. Later, a cambium appears between the pre-existing tissue and this newly formed parenchyma (fig. 5, plate 41). This cambium grows actively and forms xylem at its internal surface, toward the original explant, and phloem toward the outside.

Again, a cambium may be formed at the boundary between a phloem and the proliferating tissue that surrounds it. This is the case, particularly, in cultures of xylem of the Jerusalem artichoke made in the presence of auxin. The submerged portion produces a diffuse cambium (fig. 4, plate 40) in the midst of which phloem bundles differentiate, and a cambium layer then forms at the internal face of each bundle. At the face toward the interior of the explant, this cambial zone produces tracheids; toward the outside it gives rise to phloem, which is thus added to the original phloem bundle (fig. 6, plate 41).

If the original explant consists of phloem, the sequence is identical, but the cambial zone appears on the outer margin of the newly formed phloem bundle; that is, away from the initial explant; the phloem is so oriented that the xylem is turned toward the outside and the phloem toward the inside, toward the original phloem tissue (fig. 7, plate 42). Cambium zones may also arise in a third way between a phloem mass and a mass of tracheids nearby. This occurs, for example, in the deeper parts of a fragment of carrot phloem cultivated in the presence of auxin. The companion cells divide actively and give rise to meristematic nodules (fig. 3, plate 40) that differentiate to form tracheids at the center and phloem outside; finally a cambium is organized between the tracheids and the phloem (fig. 8, plate 43). This cambium forms many tracheids on the inside and phloem elements on the outside.

If the explant is a fragment of xylem of Jerusalem artichoke, one obtains an analogous phenomenon from the secretory canals but, in this case, the xylem is toward the outside and the phloem on the inside (fig. 9, plate 43).

We should note, finally, that a vascular cambium may arise directly in the midst of a proliferating tissue. This occurs in fragments of chicory xylem cultivated *in vitro*. The peripheral regions of the explant proliferate to produce a parenchyma in the midst of which a vascular cambium is formed.

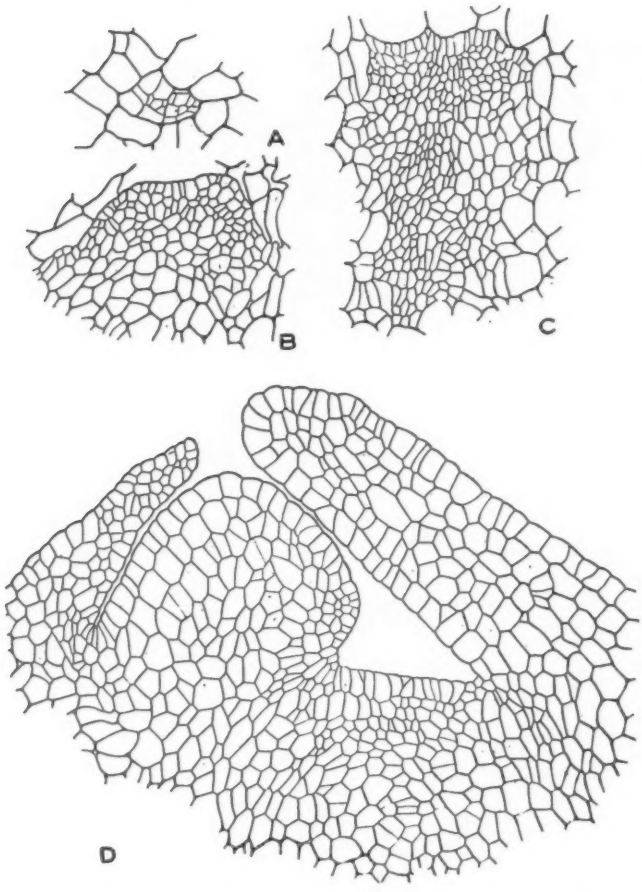
If we compare these different processes by which vascular bundles are formed, we note that they all occur in such a manner that the phloem and xylem are separated by a cambium. The particular orientation of these structures is conditioned by the nature of the pre-existent tissues. It appears that these pre-existent tissues establish, in the cells arising from them, a gradient of differentiation that is expressed in the formation of 2 poles; one situated near the pre-existent tissue and of the same nature; the other placed toward the exterior and of a nature opposed to that of the pre-existent tissue.

The formation of cambium from parenchymatous elements results from a dedifferentiation. Although they may have the same general organization as the parenchymatous elements from which they are derived and may contain large vacuoles, they are nevertheless poor in paraplasmatic products and their plastids are undifferentiated (text-fig. 2*C*).

Now let us examine the production and proliferation of vegetative growing points. Isolated bits of plant organs frequently produce vegetative growing points such as root meristems or bud meristems. Buvat, who first studied simultaneously the histological and cytological aspects of this phenomenon, noted that these growing points result from a dedifferentiation of a very small number of either parenchymatous or cambial cells. These cells divide rapidly (text-fig. 3*A*). A small number of cells gives rise to a growing point that rapidly organizes into a bud or a root meristem (text-fig. 3*B*, *C*, *D*). In this case the proliferation is always accompanied by organization. Once the growing point is established, it develops into an organ unless inhibited by the cultural conditions; this organ then tends to become morphologically and physiologically autonomous. If we follow the evolution of the cells during the organization of a root or stem growing point, we note that they undergo a progressive series of changes. The cells of a growing point have very large nuclei (text-fig. 2*D*); the plastids do not elaborate products, and the vacuoles are small. The difference between this kind of cell and other cells is in the size and form of the vacuoles. This type of cytological structure generally occurs *only* in growing points and seems to be really inseparable from this type of organization. We have, however, noted one exception, which occurs when a bit of carrot or chicory phloem is treated with high concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthoxyacetic acid. This auxin stimulates an intensive proliferation that results in the formation of very small cells associated in unformed masses (fig. 10, plate 43); but there is not a complete regression because these small cells possess less numerous and larger vacuoles than those of growing points.

Now let us turn to established strains. Histological examination of such established strains shows the same 3 types of proliferation as in initial explants. Colonies that have undergone numerous subcultures are all made up of a parenchymatous mass that may contain vascular bundles and in some cases root or stem growing points. In most cases their growth results largely from proliferation of the fundamental parenchyma. This can be seen for example in an old colony of *Amorphophallus* parenchyma. Each parenchymatous cell divides and gives rise to a nodule growing inside the original membrane and each of these cells redivides in the same manner (text-fig. 4).

Cambial proliferation and differentiation of vascular bundles also occurs in several ways. For example, in lilac and Boston ivy (*Parthenocissus tricuspidata*), the initial colonies may be entirely parenchymatous, but this state does not persist; after a few transfers, fibrovascular bundles are produced. These bundles consist of phloem associated with xylem. More commonly, colonies are, from the start, made up of parenchymatous



TEXT-FIGURE 3.—Organization of a bud meristem (after Buvat). Such a meristem is initiated by the division of a small number of parenchymal cells (*A*). These divide further (*B*, *C*) in rapid sequence and become organized into a definite bud (*D*) or root (not shown).

masses containing phloem and tracheal elements associated in vascular bundles (fig. 11, plate 44). This occurs in carrot tissue.

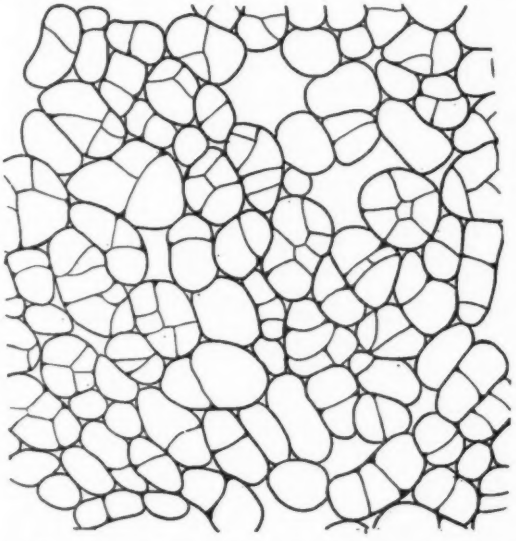
New vascular bundles in subcultured colonies are generally formed in much the same way as in the initial explants. These vascular systems may present the aspects of true bundles (fig. 11, plate 44) or of nodules. Often the associated parenchyma separates with the enclosed nodule so that the colony becomes divided into granules of tissue that separate at the least contact. Parenchymatous elements may also lignify to form islets of completely independent tracheids.

Some colonies contain tiny groups of phloem cells that are not transformed into vascular bundles. Other colonies are originally heterogeneous

but their vascular bundles are gradually eliminated and finally, upon repeated transfers, they become entirely parenchymatous. This occurs in the colonies of willow. Still other strains that at first produced vascular elements evolve into a completely parenchymatous state, but the newly formed tissue may be made up of two distinct types of elements: 1) rounded and completely isolated parenchymal cells of a high degree of functional differentiation and capable of making starch (fig. 12A, plate 44); 2) groups of very small elements without starch or other paraplasmic substances but with large vacuoles (fig. 12B, plate 44). The latter cells thus possess the cytological characters of cambial elements but instead of being assembled into a dividing layer they are grouped in masses without distinct form. We believe that these are, in fact, cambial cells which have lost the power to produce vascular elements and which give rise only to parenchymal cells.

Finally, let us examine the formation and proliferation of growing points in established strains. Root and stem growing points are both formed in such strains in exactly the same way as in initial explants; that is, they are derived from small groups of cells whose proliferation has increased at the same time that they are being organized into definite organ primordia. This is true of cultures of chicory, *Amorphophallus*, certain varieties of carrot, tobacco, and others.

In these various examples, the formation of cells of a primary meristem type is generally inseparable from the organization of growing points. But there exists an exception to this rule in the strain of tissue from



TEXT-FIGURE 4.—Section of a culture of *Amorphophallus rivieri* after repeated passages. Here one sees the mass of fundamental parenchyma. Each cell has divided repeatedly to form a small nodule contained within the original cell membrane. Each cell of this nodule in turn behaves like the original cell and again divides.

Pinus strobus isolated by Morel. The colonies have a green color and are made up of rounded masses that break up at the least contact. Microscopic examination shows (fig. 13, plate 44) that they are made up of isolated, rounded cells whose diameter may reach 100 to 200μ; among these cells are distinguished islets made up of groups of a few dozen cells, 25 to 35μ in diameter. These cells frequently contain quite normal mitoses. It has been possible to count the chromosomes in equatorial plates. There are 24, which corresponds with the diploid number for the species. These meristematic elements are rich in cytoplasm, have large nuclei, undifferentiated plastids, filamentous or granular vacuoles, and contain no starch (text-fig. 5).

The cells that arise from these centers of mitotic activity grow and differentiate very rapidly and form parenchymatous elements, which are isolated from one another and which apparently do not divide further. These have large vacuoles and their plastids are packed with starch (text-fig. 5); some even contain tannins. These tannin cells tend to be grouped together, hence derived from single cells, indicating that the capacity to form tannin may be a hereditary characteristic. Some cells may also differentiate into tracheids (text-fig. 5). These tracheids are rare in young actively growing cultures but become numerous in old ones. When completely differentiated, they are without nuclei, although we have noted nuclei in mitosis during the process of tracheid differentiation. These mitoses are often aberrant with clumping of chromosomes or with tetraploid metaphases. In regions containing many tracheids, the nuclei may be pyknotic.

This type of organization warrants particular attention, for it is contrary to the general rule that cells of a primary meristematic type are always grouped into organized vegetative growing points such as root or bud meristems. In this case, we have growing points that have *no* organization.

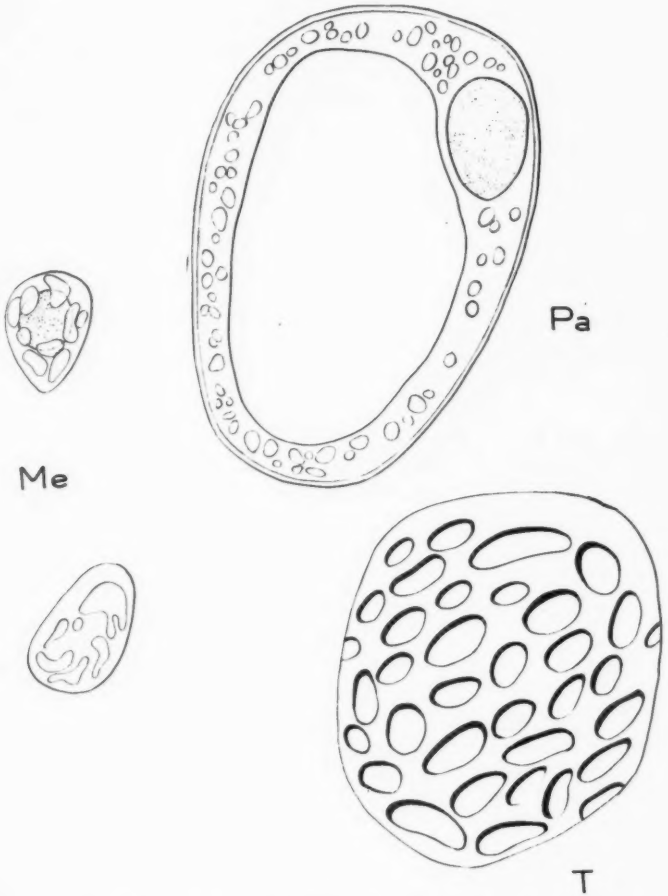
Having outlined briefly the essential features of histogenesis *in vitro*, we may consider the general conclusions that this study suggests.

Certain processes observed in primary explants are comparable to those seen in normal ontogenesis. One finds the reorganization of a cambium between two tissues of different nature both *in vitro* and in normal developing organs. The vascular cambium appears in dicotyledonous roots between the protoxylem and the protophloem, while in the stem it is formed within the procambial strands, either directly or in contact with bundles of phloem differentiated from these strands.

The method of organization of vascular bundles is likewise identical *in vitro* and in normal organs. The same is true of the differentiation of growing points. This takes place in isolated tissues in exactly the same manner as in intact plants or in cuttings.

In contrast, the parenchymatous proliferation which is so frequent in tissue cultures is extremely rare in intact plants, limited as it is to the formation of tubers, galls, and similar special structures. The formation of diffuse cambiums, which occurs often *in vitro*, is observed *in vivo* only in certain Monocotyledones.

In established culture strains we again find certain normal processes of histogenesis noted in primary cultures. The development of cambium and of fibrovascular structures is similar. Similar anomalies are also found in both. The phenomenon of parenchymatous proliferation plays



TEXT-FIGURE 5.—Principal types of cells observed in cultures of *Pinus strobus*. *Me*–meristematic cells with small, filamentous, or droplike vacuoles. *Pa*–parenchymatous cell with large vacuole and whose plastids have formed starch. *T*–tracheid.

an important role in the development of subcultures. But established strains show still more important anomalies in which the histogenic and cytological processes are dissociated. In particular, cells of primary and secondary meristematic type are often assembled in formless masses instead of being associated in cambiums or strictly organized vegetative

growing points. This anomaly does not interfere with the maintenance of their fundamental morphological and physiological characteristics.

May I add that in this brief exposition I have left untouched the phenomena of cellular hypertrophy of a hyperhydric type and the anomalies produced by heavy doses of auxins or of certain inhibitory substances, such as maleic hydrazide or *N*-1-naphthylphthalamic acid. These are not truly characteristic of cultures *in vitro*.

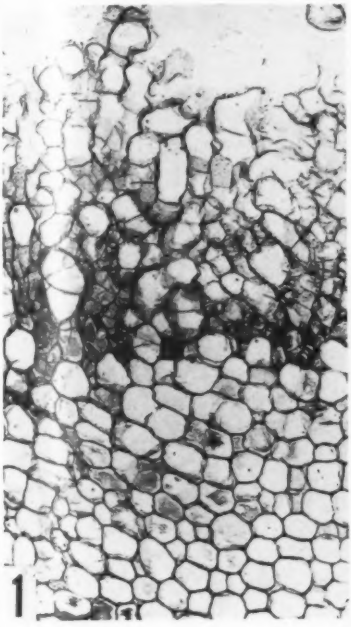
PLATE 40

FIGURE 1.—Section of a fragment of carrot phloem cultivated in a medium lacking growth substances. It has multiplied along the exposed surface to produce a thin layer of homogeneous parenchyma.

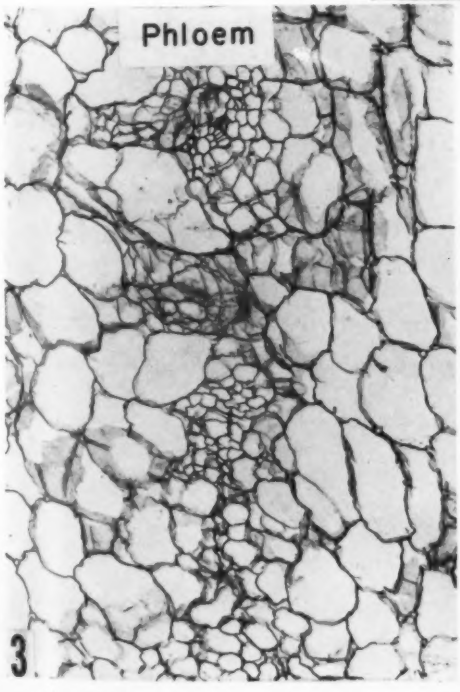
FIGURE 2.—Section of the normal phloem of carrot. It is made up of a ground parenchyma traversed by strands of small cells, the phloem strands. These are made up of sieve tubes, companion cells, and parenchymatous elements. There is also a secretory canal.

FIGURE 3.—Structure of a bit of carrot phloem cultivated in a medium containing 10^{-7} indoleacetic acid, showing a phloem strand that has proliferated.

FIGURE 4.—Section of a bit of parenchyma of Jerusalem artichoke, after a few days in culture, through the region in contact with the substratum. *Note* peripheral cells have divided parallel to the surface of the explant, forming regular files of cells thus constituting a sort of diffuse cambium.



Phloem
Secretory canal


Phloem

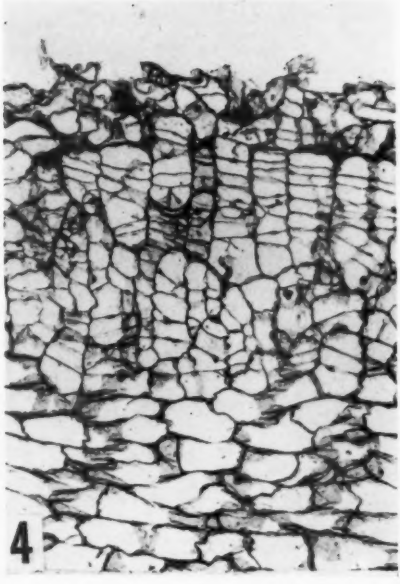
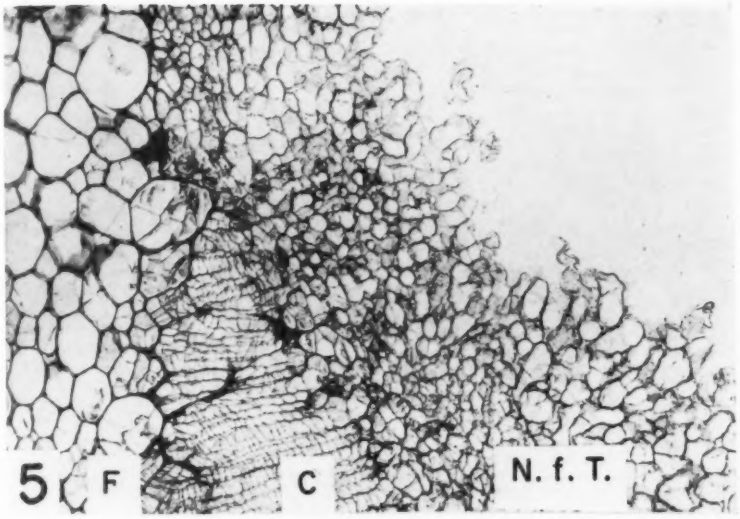


PLATE 41

FIGURE 5.—Section of a bit of carrot xylem after 59 days in culture. A cambial zone (C) has been formed between the newly formed homogeneous parenchyma (N.F.T.) (*cf.* fig. 1, plate 40) and the pre-existent cells of the initial explant (F).

FIGURE 6.—Section of a bit of xylem of Jerusalem artichoke cultivated in a nutrient containing 10^{-7} indoleacetic acid. This section is through the newly formed region. The vascular strands are made up of tracheids, centripetal to islands of phloem, from which they are separated by a cambium (C).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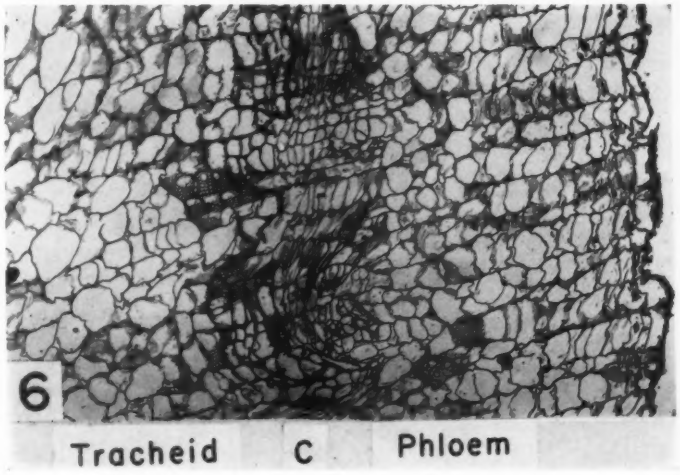
F
C
N. f. T.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Tracheid
C
Phloem

PLATE 42

FIGURE 7.—Section through a bit of carrot phloem cultivated in a nutrient containing 10^{-7} indoleacetic acid. The newly formed tissue contains vascular strands made up of tracheids toward the outside and islets of phloem toward the inside. This arrangement is instable; a cambium will later arise on the outer margin of the strands of tracheids.

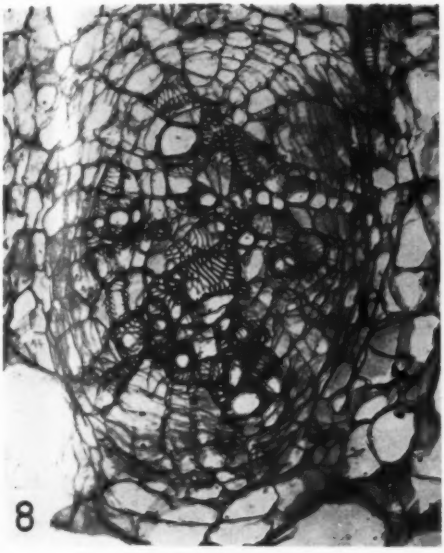
7

PLATE 43

FIGURE 8.—Vascular nodule formed in the interior of a bit of carrot phloem. This nodule was differentiated from a phloem strand whose cells first formed an islet of meristematic cells (*cf.* fig. 3, plate 40) whose central cells became tracheids and whose outer cells became phloem. A circular meristematic zone then appeared between these 2 tissues.

FIGURE 9.—Section through a bit of xylem of Jerusalem artichoke cultivated in a medium containing 10^{-6} indoleacetic acid, showing the results of proliferation of a secretory canal that has given rise to a meristematic nodule whose center has then differentiated into phloem, the peripheral part becoming tracheids.

FIGURE 10.—Section of a fragment of carrot phloem cultivated in a medium containing 10^{-7} naphthoxyacetic acid. Proliferation has given rise to very small cells that instead of being grouped into organized growing points are distributed in a uniform, unorganized mass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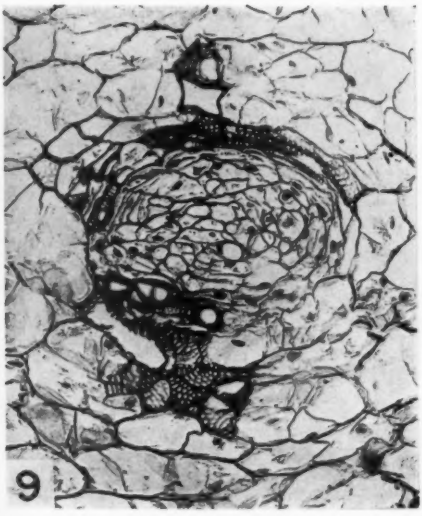
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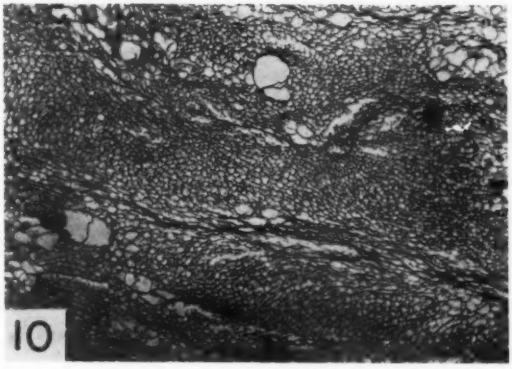
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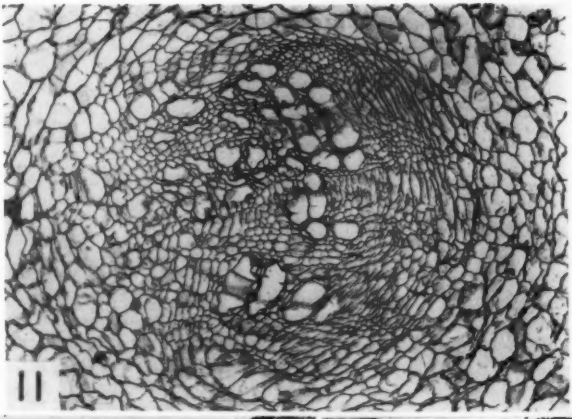
PLATE 44

FIGURE 11.—Vascular strand differentiated in a culture of carrot tissue after repeated passages. This is quite normal in organization.

FIGURE 12.—General appearance of a colony of cells from *Rubus reflexus*. At the *top* is a group of meristematic cells of the cambial type (B) with a large vacuole but with plastids that are forming no starch or other products.

FIGURE 13.—Cells of *Pinus strobus*. At the *bottom* is a group of meristematic cells, while those at the *top* are undergoing differentiation.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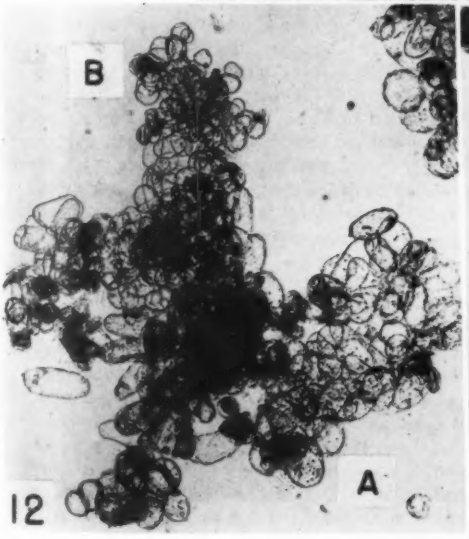
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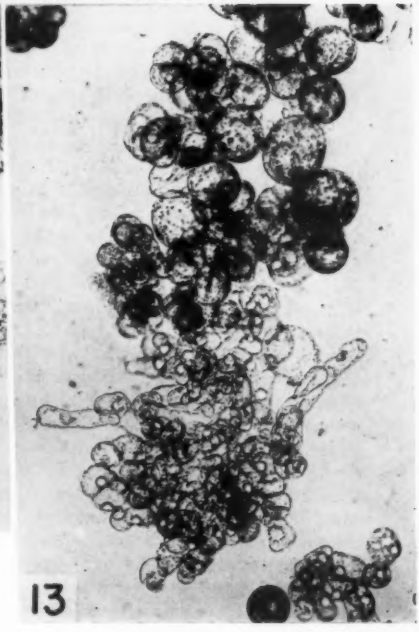
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DISCUSSION

Dr. Wetmore: Professor Gautheret has pointed out that callus tissues of various plants grown *in vitro* may develop histogenic entities, such as buds and root meristems, and even vascular tissues, at times connected with them and at times in isolated nodules or strands. It falls to the members of the panel to attempt to examine these occurrences from the morphogenic point of view, in an effort to discover whether enough is known (*a*) to account for the appearance of any of these structures in plant callus cultures and (*b*) to determine whether they can be duplicated at will *in vitro*. We have attempted to divide the field in the hope that we may thus cover our knowledge of the structures reported by Dr. Gautheret in one or another of his cultures.

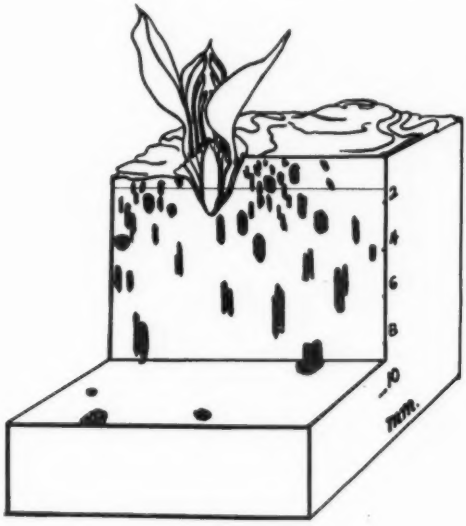
More recently, we have been concerned in our laboratories with the seemingly controlled appearance of vascular tissues in cultured cambial callus of *Syringa vulgaris*. Professor Gautheret has indicated that the initial lilac callus, even after the first few transfers, is characteristically more or less homogeneously parenchymatous, lacking vascular tissues. With this, we agree fully. But we believe we have evidence that the vascular tissues subsequently appearing in later transfers of such calluses result from our manipulation and nutrition of the callus. The evidence follows.

The technique which was first reported from Professor Gautheret's laboratory by Dr. Guy Camus and which was employed in our laboratory initially by Dr. Georges Morel has provided a method of grafting an apex of a plant into callus (fig. 1, plate 45). A V-shaped cut is made in the top of the callus, which is then filled with agar or agar containing whatever is suggested by the design of the experiment. The apex, dissected from its bud, is cut with a matching V-shaped base, which is then pushed down into the agar-filled cut. After these sterile operations, the planted callus, with its grafted scion, is planted *in vitro* on an appropriate medium and set aside for a few weeks. If grafted into callus of the same species, the graft takes readily and growth is evidenced by the apex with its new leaves, which may become separated by internodal growth; the developing scion may even develop roots.

If an experimental new callus, with a grafted apex that has grown for 50 or 60 days, is killed and fixed and sectioned (fig. 2, plate 45), the sections show that the graft union with its interdigitation may have penetrated to a depth of 3 or more mm. from the top of the callus.

If one uses agar only in the cut and the callus is planted in a synthetic agar medium of mineral salts, minor elements, 4 percent sucrose, and 50 γ per liter of naphthaleneacetic acid as an auxin, at the end of 54 days, the procambium cells of the uppermost strands on the flanks of the graft-union all developed into xylem with no phloem at all (text-fig. 1). From a level of about 2 mm. to about 9 mm. below the top of the callus, strands were progressively fewer in number, though all were comprised of xylem, or xylem and some undifferentiated elongated procambial cells and even some scarcely elongated parenchymal cells. Closer to the bottom of the callus, that is, close to the medium, strands became more numerous with a definite, preferred, vertical orientation of the elements. These elements proved to be both xylem and phloem, the latter indicated by fluorescent microscope tests for callose as well as by staining with resorcin blue (lacmoid) as indicated by Cheadle, Esau, and Gifford (1954).

If, in other experiments, one puts 4 percent sucrose and an auxin (naphthaleneacetic acid in a concentration of 50 γ per liter, as before) in the agar used in the cut, one finds, after 2 months, that nodular strands appear on the flanks of the grafted scion as before, but much more numerous. It should be mentioned that the callus after 3 or more transfers is non-polar to auxin movement so that its passage through the callus is along a diffusion gradient only. These strands now prove to possess xylem and phloem. In this case, the auxins and sugar produced by the developing scion



SYRINGA VULGARIS
54 DAYS

TEXT-FIGURE 1.—A stereogram of a piece of lilac callus into which was grafted an apex of lilac bearing 2 to 3 pairs of leaf primordia. The incision was filled with 1 percent agar containing naphthaleneacetic acid in the concentration of 0.05 mg. in per liter. This drawing was made from a study of a series of transverse sections when the graft-callus had grown 54 days. *Note* the scattered strands as seen longitudinal view. (With permission of the editor of the *Journal of Arnold Arboretum*).

are supplemented by those added to the agar. As was true in the callus near the medium in the previous experiment, the sugar and auxin prove to be significant variables, for both phloem and xylem result as earlier when sucrose at a 4 percent concentration and an auxin such as naphthaleneacetic acid at 50 γ per liter were present in the medium. When the medium contained neither sugar nor auxin but mineral salts and minor elements only in the agar, no vascular strands appeared at all (text-fig. 1) in that part of the callus close to the medium.

The judicious use of sugar, and auxin in the cut, without any scion at all, has proved to give essentially comparable results to those of the last experiment; that is, strands are produced along the flanks of the cut, strands with both xylem and phleom or xylem alone, according to the concentrations employed. Perhaps more startling was the recognition that these strands tended to form a circle, when observed in transverse section, as they do in stems, with the phloem on the outside and xylem toward the cut. If the callus is kept long enough, the individual strands in the ring may become connected by a cambial-like layer as in a stem, dividing the callus into a pseudopith centrad to the strands and a pseudocortex outside. When the medium contains sugar and auxin, the strands appearing in the callus near the medium are without any regular arrangement in contrast to what is found following the local application of an auxin-sugar-agar medium in a cut, either supplementing the auxin and sugar diffusing from a scion, or alone, when there is no scion.

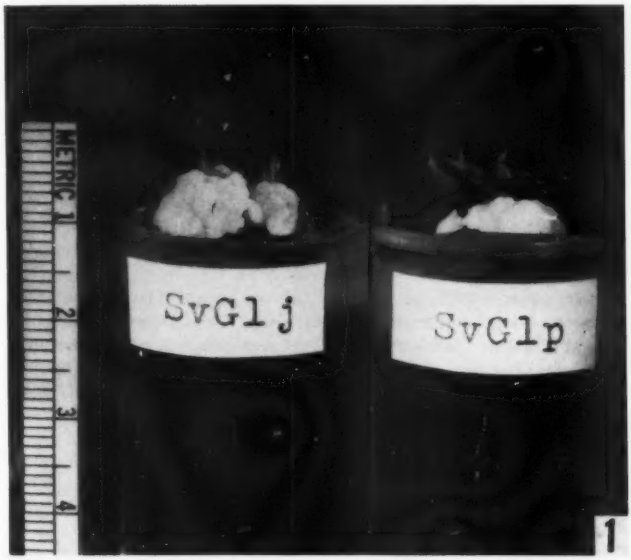
We have then a possible partial explanation of the appearance of strands in the cultured callus of *Syringa* as reported by Professor Gautheret. The work needs to be

extended and must be applicable to other calluses as well. The fact that strands with both xylem and phloem can be produced in generally predictable positions and patterns, even duplicating in considerable measure those of stems, would suggest that we may be correct in the conviction that at least two pertinent variables are a carbohydrate source and an auxin, since the varying and yet predictable results were obtained by altering the position of application and the concentration of these two substances.

PLATE 45

FIGURE 1.—Habit of *Syringa* apex grafted into *Syringa* callus, 7 weeks old.

FIGURE 2.—Longitudinal section of callus-graft union of a 5-week-old graft of a stem apex of *Syringa* into *Syringa* callus. *Note* black-stained agar around the base of the scion. Two lobes of the scion are shown on *left*, the *upper* cut almost in median plane, the *lower* in this section not shown in direct connection with the main body of the base of the scion. × 10 (With permission of the editor of the *Journal of Arnold Arboretum*.)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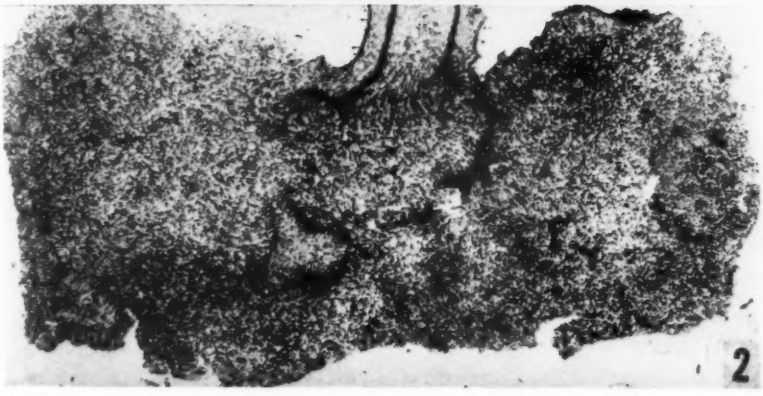
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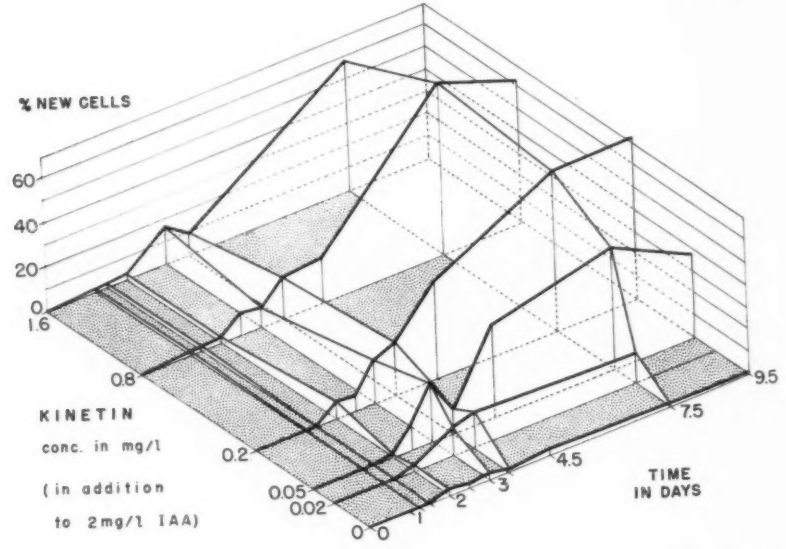
Dr. Skoog: In commenting on Dr. Gautheret's paper, I am reminded of the words ascribed to an earlier great Frenchman: "Never say no, always say yes, but." I am in agreement with what Dr. Gautheret has said almost 100 percent, but not quite. He has described numerous histological patterns or phenomena occurring in different tissues and cultures of different species. What he has said, no doubt, is true but only under certain specified or, rather, certain as yet unspecified conditions. It would be a serious error to believe or to imply that some definite, meaningful relationship exists in the associations he has described between different kinds of histological manifestations and the particular tissue cultures of different species in which these were observed. I would like to point out that the aspects of histogenesis he has dealt with and, furthermore, all the aspects of morphogenesis, *i.e.*, the initiation and development of organs and whole plants from callus, may be obtained from one type of tissue culture from a single species by proper manipulation of growth factors in the nutrient medium. My thesis is that plant growth and differentiation of all types are determined by quantitative levels and proportions of various growth factors. I hope you will excuse the term, "growth factor," as it is necessary for me to use it. It merely refers to one of the numerous organic substances, other than energy furnishing substrates, that are required for growth of plant cells.

I shall be concerned mainly with two types of growth factors, which seem to be particularly limiting in the growth of plant tissues, the auxins [especially indoleacetic acid (IAA)] and kinetin. The latter substance was isolated on the basis of its activity in inducing cell division and growth of plant tissues (*1*) (text-fig. 1), and chemically it is 6-*N*-furfurylaminopurine. We have found a large number of closely related substances to be similarly active. Tissue cultures of only one species, *Nicotiana tabacum*, var. Wis. #38 will be considered.

The effect on growth and morphogenesis of quantitative change in a single growth factor, kinetin, is shown in figure 1, plate 46. Note that the type of growth changes drastically from a very slight increase, soon followed by death in the absence of added kinetin, to vigorous continuous growth in its presence. With the lowest concentration,



TEXT-FIGURE 1.—Effect of the kinetin concentration and time of treatment on the number of new cells (percent dividing cells) in excised tobacco-pith tissue cultured on modified White's nutrient agar with 2 mg. per l. IAA (*2*).

there is a very marked root development; followed by undifferentiated growth of callus, prolific bud formation on the callus, and, finally, inhibition of growth as the concentration is increased.

The effects of varying levels and proportions of IAA and kinetin are illustrated in figure 2, plate 46. Note, furthermore, the effect of casein hydrolysate (fig. 2B) in promoting the development of roots and shoots, especially in the presence of high IAA concentrations, even though it is practically inactive when it is added alone to the nutrient medium. The quantitative nature of the interaction of growth factors in the regulation of bud growth (initiation and development) is more clearly indicated in figure 3, plate 46.

It may be shown furthermore (fig. 4, plate 46) that the growth-promoting action of casein hydrolysate in these experiments may be exerted by a single amino acid, tyrosine. This, of course, does not exclude contributory effects or similar functions of other components in the hydrolysate.

I believe that the above examples serve to demonstrate my point as to a quantitative regulatory mechanism in morphogenesis of plant tissues. Inasmuch as relatively minute changes in environmental conditions may influence the contents and activities of endogenous growth factors, they will undoubtedly influence histo- and morphogenesis. Therefore, it is meaningless to describe the anatomical or morphological structure of tissue cultures as if certain growth forms were characteristic of certain species or clones of plants without more detailed reference to the precise conditions and time of their cultivation.

References

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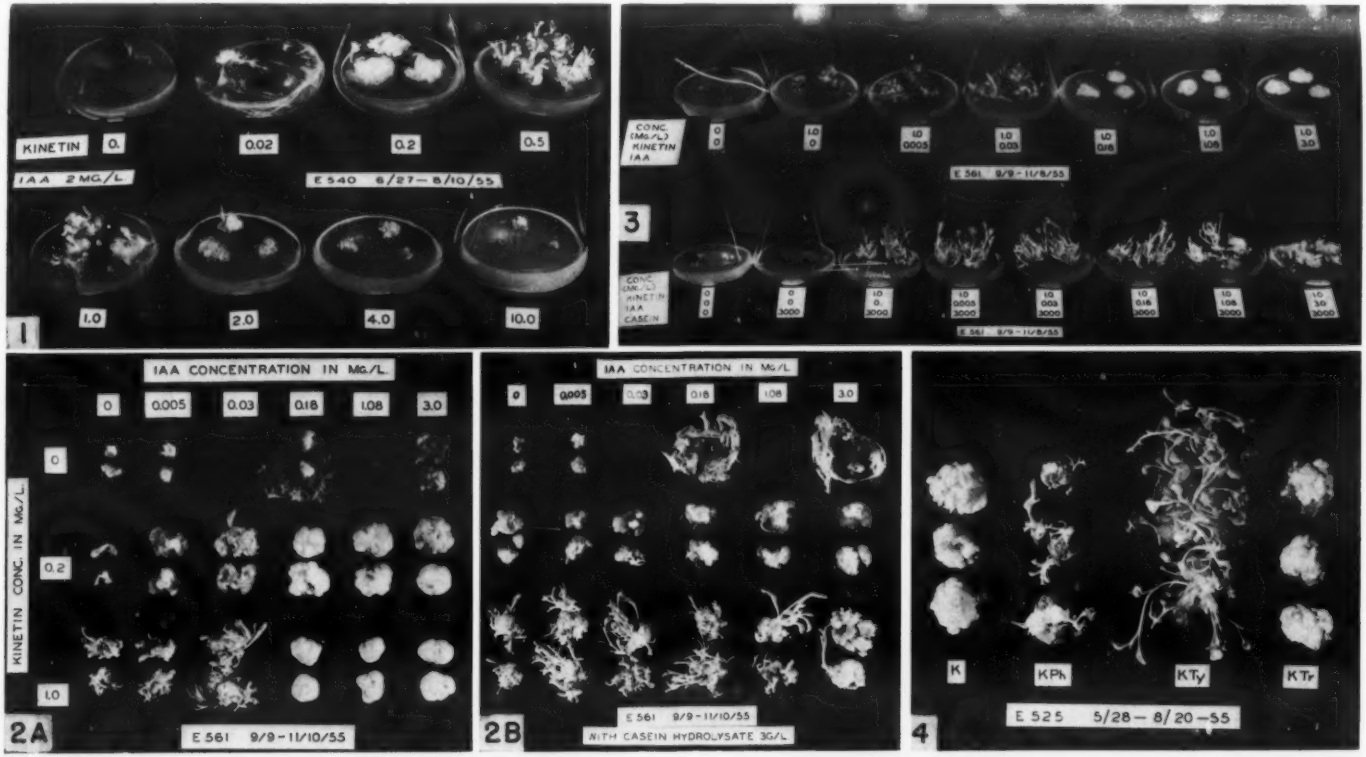
PLATE 46

FIGURE 1.—Effect of kinetin concentration (in 0-10 mg. per l. range) on growth and bud formation in tobacco callus cultured on modified White's nutrient agar with 2 mg. per l. IAA added. Age of cultures 44 days (*3*).

FIGURE 2.—A and B. Effects of different levels and proportions of IAA and kinetin on growth and organ formation of tobacco callus cultured on modified White's nutrient agar: *a*) without, *b*) with 3 gm. per l. casein hydrolysate added. Age of cultures 62 days (*3*).

FIGURE 3.—Effect of increasing concentration of IAA in the presence of a relatively high (1 mg. per l.) kinetin level: *upper*, without, and *lower*, with 3 mg. per l., casein hydrolysate in the medium. Age of cultures 62 days (*3*).

FIGURE 4.—Effect of amino acids on growth and organ formation of tobacco callus cultured on modified White's nutrient medium with 2.0 mg. per l. IAA and 0.2 mg. per l. kinetin added in each case. Addenda *left* to *right:* K, none; KPh, DL-phenylalanine 150 mg. per l.; KTy, DL-tyrosine 210 mg. per l.; and KTr, DL-tryptophan 60 mg. per l. Age of cultures 84 days (*3*).


KINETIN 0. 0.02 0.2 0.5
IAA 2 MG./L
E 540 6/27 – 8/10/55
1.0 2.0 4.0 10.0
1
CONC. (MG./L.) KINETIN IAA
E 561 9/9 – 11/8/55
3
CONC. (MG./L.) KINETIN IAA CASEIN
E 561 9/9 – 11/8/55
IAA CONCENTRATION IN MG./L.
0 0.005 0.03 0.18 1.08 3.0
KINETIN CONC. IN MG./L.
0 0.2 1.0
2A
E 561 9/9 – 11/10/55
IAA CONCENTRATION IN MG./L.
2B
E 561 9/9 – 11/10/55
WITH CASEIN HYDROLYSATE 3G/L
K KPh KTy KTr
4
E 525 5/28 – 8/20 – 55

Dr. Ball: A number of problems of morphogenesis can be studied by use of the *in vitro* or sterile culture technique. We take advantage, especially, of the growth of mature parenchymatous cells as Professor Gautheret has so admirably shown, deriving them from the tissues of the indeterminate meristems. As most of you know, the internal tissues of a bud or the tissues at the tip of a root of an angiosperm are primordial meristems, that is, meristematic tissues that have an indeterminate type of growth. They never mature, never show the characteristics of cytology, or of differentiation, or of external form, which are characteristic of mature tissues. The measurements of cells in these regions are extremely small. Inside the bud of a lupin plant or the lilac plant, the meristem, the dome of extremely young cells, would be perhaps 100 or 200 μ across. By means of microsurgery, it is possible to excise these tissues and grow them in culture. Their growth in sterile culture is very different from that of parenchymatous tissues from carrot root or some tissues of *Amorphophallus* stems or roots, chicory roots, etc. In the latter the parenchymal cells lose their polarity and show a new system of growth in culture. The primordial meristems of root and shoot retain their polarity and grow in an approximately normal fashion, indicating that they have the ability to produce the whole plant. This is of some interest because it shows a completely different level of differentiation of the inoculum.

The material lends itself to interesting studies in nutrition. Whereas the parenchyma, in many cases, requires growth factors and vitamins of various sorts to carry on growth, the meristematic tissues of the bud or the root tip can be grown in some cases, probably most, without anything except sugar and water and appropriate minerals. The nutritional studies in this regard are very instructive. From the angiosperms we can grow a bud that has an apex, 2 or 3 foliar primordia, and a little bit of subjacent tissue. But if we attempt to take out the primordial meristem itself, about 300 μ across, we can't grow it, indicating that the physiological differentiation has perhaps progressed to the degree, in the angiosperms, that foliar primordia, even at this young stage, furnish something to the apex that will permit it to grow. However, if we take the vascular cryptogams and at least one gymnosperm, we can dissect out the apex and grow it by itself, and the few hundred cells of the apex produce a whole plant. This is indicative, perhaps, of differences in the level of physiological differentiation in the higher plant tissues. If we couple this study with experimental morphology of the shoot apex itself on the intact plant, if we isolate the shoot apex by 4 longitudinal cuts within the zone of foliar primordia, it will grow into a perfectly normal shoot nourished by substances that come up through the pith. This indicates that the plant produces something that we have not duplicated in sterile culture media. The same sort of thing has been done on root tips of pea by Dr. Torrey at Berkeley. He took root tips ½ mm. long (500 μ) and grew them into an entire root system in sterile culture. Furthermore, in the normal plant, we can divide the shoot apex by longitudinal incisions so that one fourth or even one sixth of the apex will produce, eventually, a normal shoot. But that can't be accomplished yet in sterile culture, indicating that we are a long way from really understanding how this growth process goes on. There is a great need for further studies of the biochemistry of this kind of growth.

One other thing about primary meristems that should be brought out here, and I think Professor Wetmore is the leader in this, is their organizing effect—their function as organizers in much the same sense as the experimental embryologist of animals have used the term. For example, as Camus showed, and Professor Wetmore also, the bud, when grafted into a callus, determines the formation of procambium and, eventually, vascular tissues in that callus. The same thing happens in a callus culture, either a new one or one that has grown for some time and has undergone several transfers in the sterile culture medium. The bud, when regenerated, produces vascular tissue behind it, as though the bud were secreting certain substances that move in a definite direction in the callus. And this can be substituted in certain cases by certain growth substances such as indoleacetic acid. The problems of morphogenesis, I think, have been definitely assisted by utilization of the sterile culture procedure.

Dr. Steeves: I should like to comment briefly on the culture of plant parts of another category, which differ from those discussed by Dr. Ball in that they are determinate in their growth. Whereas the shoot apex and the root apex, both on the plant and in culture, are capable of indefinite development, other organs such as fruits or leaves are defined as organs of determinate growth; that is, they typically undergo an active period of development following which they attain a complete maturity and undergo no further growth.

In the realm of fruit culture, Nitsch has carried out extensive studies of fruit development *in vitro*, from unopened flower buds to mature and even ripe fruits. He has investigated the physiological factors involved in this process of development and particularly the role of growth hormones in the growth of these fruits with and without pollination.

In a similar vein, Dr. I. M. Sussex and I have been studying the development of leaves. Leaves are typically developed, as Dr. Ball has mentioned, from primordia that grow out near the base of the apical meristem. These primordia at first do not differ much in appearance from the meristem that produces them; but they very soon manifest quite different properties in that they acquire a dorsiventral symmetry and, in contrast to the meristem that produces them, undergo development only for a limited period of time. The point which interests us in this matter, and which has been of interest to botanists for many years, is to know exactly how this comes about. For initial studies we used *Osmunda cinnamomea* L., the common cinnamon fern, and we excised leaf primordia of a variety of sizes and ages from the compact bud of this plant. The youngest and smallest were less than 1 mm. in length, the largest perhaps in the range of 2 cm. In the initial experiments, we did not include the most recently produced primordia at the shoot apex. On a relatively simple medium containing, in addition to agar, only water, mineral salts, and sucrose, these primordia underwent complete development and produced mature leaves (fig. 1, plate 47). This involved a rather complex development including apical growth of the leaf, the formation of a crozier, the uncoiling of this crozier, and the formation of leaflets or pinnae that were not present in the initial explants. All of this complex process went on in complete isolation from the plant, indicating that, once it has become established in the primordium, it is inherent within the leaf and is not dependent upon continued attachment to the plant. The only thing required is that the leaf be adequately nourished. In some way these characteristics are imposed upon a tiny mass of meristem in the region of the shoot apex. The mature leaves produced were, of course, considerably smaller than those found on the natural plant, but they were typical of the species. We subsequently extended these studies to the angiosperms, and we have obtained a similar result with several species. Figure 2, plate 47, shows 3 leaves of *Helianthus annuus* L. grown to maturity *in vitro*.

We next turned our attention to the culture of the latest primordia that have been produced, those in the immediate vicinity of the apex. These are barely visible mounds of meristematic tissue that, to all appearances, do not differ significantly from the meristem that produced them. When we excised these primordia and, in particular, the 5 primordia closest to the apex, we found that their development differed considerably from their earlier formed predecessors, which we had studied in the previous experiments. In almost all cases, these young primordia, which were extremely minute at the time of excision, did not develop into leaves, as the older primordia had done, but into complete plants (fig. 3, plate 47). Initially a shoot developed and then roots formed. We thus obtain, from these primordia, not typical leaves but entire plants. We may conclude that these primordia, in the first stages of their growth, have exactly the same potential as any other part of the apical meristem. It is apparent that these tiny masses of meristematic tissue, which are destined to become leaf primordia if left alone, at an early stage have not acquired that potentiality, at least not irreversibly. We concluded that one can recognize and, in fact, bring into sterile culture, leaf primordia, at a stage before their determination has become complete. We feel that this is a matter of some significance, because it does open up to the techniques of sterile culture the study of organ determination

in plants, since we can excise primordia and put them into culture before determination has become irreversible. Therefore we may hope that it will be possible to experiment with this process of determination and ultimately to decipher some of the factors that bring about such a complete change in the potentialities of a tiny portion of meristem developing on the flanks of the apical meristem of the shoot. This work has been done with ferns, and we have not as yet been able to culture primordia of this order of development from the angiosperms. We have, as has been shown, cultured older primordia, and we hope soon to be able to extend this to the younger stages.

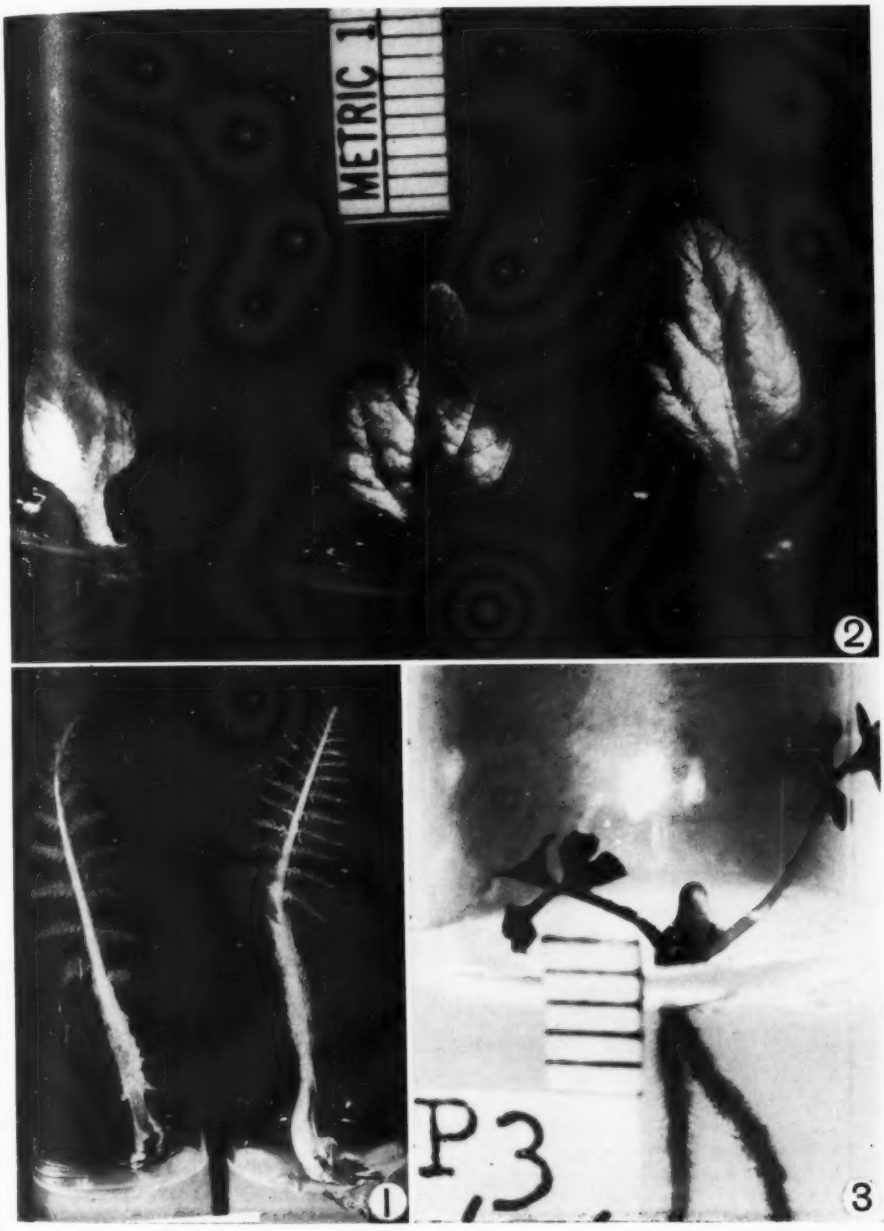
The purpose of these descriptions has not been particularly to report a specific set of experiments but rather to show that the technique of sterile culture, about which we have been hearing so much in its application to both plants and animals, can also be applied to the study of the development of determinate organs in the vascular plants.

PLATE 47

FIGURE 1.—Leaves of *Osmunda cinnamomea* grown *in vitro* from the primordial stage to maturity. × 1

FIGURE 2.—Leaves of *Helianthus annuus* grown *in vitro* from the primordial stage to maturity.

FIGURE 3.—A small entire plant of *Osmunda cinnamomea* developed *in vitro* from third primordium (P_3) of a shoot apex.


METRIC 1
2
1
P,3
3

Dr. Steward: Dr. Steeves, in isolating these very young primordia and developing a whole plant from them, I take it there was no suspicion of an axillary bud at the base of the leaf?

Dr. Steeves: Actually, I feel quite confident in regard to the particular fern that we used, because we have never found evidence of axillary buds in any stage of development and we do not find detached meristems.

Dr. Steward: I thought these were angiosperms.

Dr. Steeves: No, the leaf primordia that developed into whole plants were from the fern. We do not find any evidence of the detached meristems about which Wardlaw has written in *Dryopteris*. And they do not develop in any later stage.

Dr. Lwoff: I would like to ask Dr. Skoog if the kinetin has to be present continuously during the experiment, and what happens if it is suppressed after a few hours, 1 or 2 days, or a week?

Dr. Skoog: Yes, the kinetin has to be present to be effective. We haven't tested for this at 1 day, 2 days, etc., but tissue may be cultured for months on medium with kinetin and when transplants are then removed to a non-kinetin medium, they will not grow. There are some very interesting experiments on the morphogenesis in mosses, by Eakins and Girton, at the University of Texas, with kinetin that gave results similar to ours, except that they were dealing with branching of mosses rather than bud initiation. Apparently they were able to demonstrate the separate action of IAA and kinetin by first giving the auxin for a few days and then the kinetin. Both substances are required for growth, but there may be intermediary substances between them, *i.e.*, they need not necessarily react directly with each other.

Dr. Fell: I was very interested in the experiments that Dr. Steeves described on the development of the complete leaf from the primordium, because it seems to be essentially the same phenomenon as we have seen in animal material in culture. For example, when we explant the mesodermal limb-bud rudiment *in vitro*, it will form a miniature limb skeleton consisting of a pelvis, femur, tibia, fibula, and, perhaps, the metatarsal region, and all developing in response to factors intrinsic in the primordium. This is exactly the same type of self-differentiation as that shown by the leaf rudiments *in vitro*.

Dr. Morel: I would like to comment on Dr. Skoog's statement. Like my famous countryman, Aristide Briand, I agree with 90 percent that you said *but*, if the same experiment is done with kinetin and auxin and instead of using tobacco tissue, which normally produces buds, a homogeneous callus of *Parthenocissus* is used, which normally never produces buds, it is impossible to bring about bud formation with kinetin. I do not think that Dr. Skoog has described a very general phenomenon. In some other species there may be other mechanisms regulating bud formation.

Dr. Skoog: I don't even say that kinetin, alone, works in all kinds of tobacco. For example, Dr. Tryon has developed strains from the same origin and grown them for several years as undifferentiated callus. All of a sudden, some of these cultures produced buds and continued to form buds in subcultures for years. Other cultures of the same material remained undifferentiated and were subcultured for another two years, after which they started to produce roots and have continued to do so in subcultures. These substrains are different and react differently. However, it is possible to counteract bud formation in the budding strain with indoleacetic acid added to the medium, and to counteract the root formation in the rooting strain with added kinetin. There are still other cases in which neither kinetin, adenine, nor tyrosine, added alone, will lead to bud formation, but combined treatments with the three substances are effective. The whole point of my argument is that it isn't one substance but it is the quantitative interaction of all the factors required for growth. If you say, therefore, that kinetin probably occurs and acts in tobacco but that the results have no reference to other species, I cannot agree.

Dr. Morel: I mean that this phenomenon of bud formation from a differentiated cell is a special property of the species, shall we say, of the clone of the cell that you are studying.

Dr. Skoog: No, I don't believe that. I believe it is largely a reflection of the growth-factor composition. After all, the formation of shoots is not restricted to a few plants. The fact that it is obtained with kinetin in such different material as mosses and tobacco makes me think that the mechanism is a pretty general one.

Dr. Morel: Don't you think that in other plants there may be other regulating mechanisms?

Dr. Skoog: Any factor that may limit growth can be a regulating factor in a particular case, but this would not necessarily mean that the mechanism of bud formation is different in each case.

Dr. White: I think that what we have to do, essentially, is to go back to Claude Bernard, as was emphasized by Carrel. We have to consider the total system of which the explant or the organism is only a part, and the surroundings, a part. Once we get down to the level in which we can integrate those two, we are going to find pretty general phenomena, but in which some tissues contribute one thing and others contribute another. In the total picture the environment will also contribute different things to build up that generalized pattern.

Dr. Gautheret: I want to point out that the study of the organ formation requires the use of specialized tissue of certain species because most of them are not capable of giving rise to organs. When a tissue is not capable of spontaneously producing organs such as roots or buds it is not possible to promote this formation by experimental means. Tobacco tissue, chicory tissue, and *Scorzonera* tissue are normally capable of giving buds. These tissues can be further stimulated experimentally. But other tissues, for example, the willow tissue, do not produce buds or roots, and no one has yet been able to obtain organs from these tissues experimentally. In the case of the Virginia creeper, there are two species, *Parthenocissus tricuspidata* and *Parthenocissus quinquifolia*. The first never produces organs, the second, very frequently, produces roots. With auxin, the second produces more roots than normally, but the first still produces none. As Dr. White has said, a particular species can be used for a special purpose, but it is not possible to expand facts obtained from one kind of tissue into a general theory.

Dr. Woolley: I would like to ask Dr. Gautheret if the meristem has the capability, in contrast to other parts of the plant, of making these various growth factors. Can you, Dr. Gautheret, make an extract of the meristem of willow and make willow cultures form organs?

Dr. Gautheret: This attempt was not made, but your question must be more generalized. I think that we have two series of facts. One series concerns the histological point of view; another series concerns the physiological point of view. We must try to correlate these two points of view.

Dr. Skoog: In the willow this has not been done, but in tobacco it is possible. Leaf extracts can be substituted for kinetin to give these effects. With reference to Dr. Gautheret's statement that we cannot generalize from experiments on one species, I would like to point out that chemically induced morphogenetic effects of the type I have discussed have now been reported for widely divergent types of plants.

Dr. Ball: One series of experiments on tissue culture has been found by a number of workers to be pertinent to this question of bud formation. If one takes an original explant from a plant such as *Sequoia sempervirens*, with which I have worked a great deal, and grows it on sterile culture medium for a period of 2 months and then cuts it into small bits and transfers it repeatedly, for the first 4 transfers the tissues form buds abundantly, but, after the fourth transfer, it has been my experience that bud formation falls off. One series of cultures that I have is now about 8 years old and never forms buds under any circumstances. For a while, I thought it was possible to make it form buds by using some of the pyridines but that has not been possible. It appears, then, that possibly there are substances carried over in the original inoculum that would have a bud-forming stimulus, but those substances are lost by further accommodation to the sterile culture conditions.

Dr. Morel: I would like to point out that the stem is a very complex organ. If you try to cultivate such an organ, it is possible, even with angiosperms, to grow very

small pieces, about 100 μ, without leaf primordia by placing them in a complex medium with sulfur-containing compounds like glutathione and cell-division factors like kinetin. Such a tissue will grow and produce a perfectly normal plant. But if, instead of excising the whole stem tip, you take out that part immediately below the tip, which is the organizing center, instead of producing a normal plant it will grow in a disorganized manner and produce a callus of parenchyma exactly like callus cultures from root or from stem.

Dr. Steeves: I did not want to add particularly to this point but to the one Dr. Ball made. We have made some observations recently that are not strictly comparable in the nature of the material but seem to indicate almost the reverse process. We have been studying the phenomenon of apogamy in a species of fern: the direct development of the sporophyte from the prothallus. The prothallus is a simple sheet of cells and the sporophyte is, of course, the organized vascular plant. We have found that in early transfers, after we brought the prothalli into culture, we do get this phenomenon. It is not until we have had them in culture for several years through perhaps 10 or 12 transfers that they begin to acquire the ability to produce the vascular sporophyte directly, which I think would be comparable to the production of a bud from this rather simple prothallus. Whether this is the acquisition of something that had not been present, I do not know. We hope to find out.

Dr. White: I brought back some of Dr. Gautheret's callus cultures from France in 1949. At that time, he told me they had lost the capacity to produce roots. During the first year I had them, they very frequently produced excellent roots but have not done so in the last 6 years. There was some difference in our method of handling them during that first year from what Dr. Gautheret had been using and from what we have subsequently used. We have no idea what that difference is.

Dr. Thimann: A small experiment Miss Wickson and I have done suggests, so far as chemical control is concerned, there is perhaps not so great a difference between the initiation and the subsequent development of an organ. If one cultures isolated buds of peas with attached internode tissue, then under the right nutritional conditions the bud develops, elongates, and eventually produces leaves. If auxin is added to the solution, this bud elongation can be prevented exactly as it is prevented in the normal plant by apical dominance. However, in the presence of graded concentrations of kinetin, it is possible to remove this inhibition by auxin completely so that with the right balance of kinetin and auxin, the bud develops normally. This bud was fully formed at the start of the experiment, and it is only its development, and not its formation, that is being controlled. Thus the role of kinetin in bud formation cannot be so clearly distinguished from its role in subsequent development.

Dr. Skoog: The way we found kinetin originally was by attempting to find factors that would oppose the inhibiting action of auxin on bud development. From the standpoint of regulation, I don't think there is any difference in principle between the two phenomena of initiation and further development of buds, although there may be differences in a quantitative sense.

Dr. Ball: I would like to add that the quantitative effect of various agents on bud growth or the growth of the terminal meristem or shoot in culture is a point of some interest. If one excises the piece of tissue subjacent to the bud of Lupin, about the same volume as the bud, and gives it a culture medium containing various concentrations of autoclaved coconut milk, this tissue is stimulated to grow in a rather extreme fashion. An extremely small inoculum will produce an enormous callus culture. But if one gives the excised bud itself the same culture medium, it is not affected in the same manner at all. Instead of being stimulated to form a callus, it grows to form numerous axillary branches; instead of one shoot, there may be six or seven.

Dr. Boll: I should like to ask two questions—the first one of Dr. Skoog: Have you succeeded in isolating kinetin, or any compound similar to it, from tissues under such conditions that you would not expect kinetin to be produced by the procedure? The other question I would like to ask of Dr. Gautheret. When you were talking about the cultures that were produced from the apical meristem, you said that the cells became separated from each other and you showed a tracheid. I wasn't quite

sure whether one of these tracheids was formed inside the parenchymatous mass or whether it was formed from a single cell separated from the culture?

Dr. Skoog: We haven't yet isolated kinetin from higher plant material. We have highly active extracts of something with comparable physiological action. On the other hand, it is very difficult to believe that kinetin would not be formed. If you place a bottle of deoxyribonucleic acid (DNA) on the shelf and come back a year later, you will find kinetin. If you place a bottle of wet D-oxyadenosine in the autoclave, a lot of kinetin is formed in a few minutes time. It is an extremely stable compound and as long as there is nucleic acid, DNA at least, chances of getting kinetin and/or closely related substances are very good.

Dr. Gautheret: The tracheids are not always in masses. The differentiation may take place in an isolated cell.

Dr. White: I would add that that is also the case of our spruce cultures. Isolated cells may differentiate into this type of cell. It is not necessary to have a mass.

Dr. Boll: It is also true in the lilac culture.

Dr. Steward: After this extremely interesting discussion, it seems to me that one or two further points might be made so that, at least, we don't get carried away in directions that might prevent us from making the most of some of these interesting phenomena. I find it inevitable to believe that there are these growth-promoting substances, which, in the balanced way that Dr. Skoog has described, act with indoleacetic acid or other substances to control the ability of an individual cell to divide or to reduplicate. It is inevitable that there will be many other natural substances that we shall discover which will control these processes; substances other than indoleacetic acid and substances other than kinetin. However, it also seems to me that we are still not quite clear that these regulating effects, which control the activities of cells, are enough to control the entire organization, which is, after all, the point of this discussion. I can visualize that in the environment of the organized apex of either root or shoot, these phenomena make an impact on the ability of the cell to grow by division or by elongation. I am not at all clear at the moment, and I think this is in line with Dr. Morel, that there is not something else associated with the development of the root or shoot as an organized structure. I find it a little hard to express this idea. Perhaps it is the analogue of the organ-forming bodies that we are seeking or perhaps the solution is inherent in the geometry of the actual environment of the cell in the organized growing point. I do feel quite definitely that one or two isolated observations of bud formation, where buds probably could form anyway, shouldn't blind us to the fact that we ought to keep looking for biochemical or other mechanisms, and for additional factors, that control cell division *per se*. While I feel, very definitely, that this still needs to be a primary task of the future, it doesn't in any way detract from the challenge or the stimulus of what we have heard, but it does mean that we should keep looking for factors other than kinetin and indoleacetic acid.

Dr. Henderson: Concerning a point that Dr. Steward raised, there is a question I would like to carry a bit further. It has to do with the potentiality for differentiation of the plant cell as compared with the animal cell. I think it might be agreed that the plant cell has this potentiality to a greater degree than animal cells, with their apparently very specific organizational properties. I wonder if anyone who has worked with isolated plant cells, carrying them in culture from a single isolated cell, has allowed them to coalesce or amalgamate into a tissue. Has anyone, by adding kinetin or other factors caused them to develop more than just masses of similar tissues and to differentiate into different cells or organs? Dr. Riker might wish to say how far he has carried his individual cells to amalgamated differentiated or undifferentiated tissues. This obviously would be the ideal thing to start with. This isolated cell, whatever its origin may be, could indicate whether it is necessary to have the juxtaposition of cells physically or whether these chemical factors alone can cause this differentiation into a whole plant or whole organ. I wonder if Dr. Riker would add to this point.

Dr. Riker: The cultures derived from single cells open many important avenues of research.

The technique was very simple. With aseptic technique, Dr. Muir commonly placed a small piece of sterile filter paper on an actively growing callus culture. It rapidly absorbed moisture from below. The single cell was placed on the filter paper. Commonly 8 percent, but sometimes 50 percent, grew. These plant cells had no pseudopods or dendrites to cause difficulty. The theoretical possibility that these plant cells might grow through the filter paper is opposed by various lines of evidence.

After the new clones had been established, a suitable balance between kinetin and auxin was supplied, such as Dr. Skoog has described. In a number of cases, apparently normal roots and shoots have developed. Since Dr. Morel has successfully grown plants from such shoots, all the critical steps to secure a whole plant have been taken, though not yet in sequence. To secure a normal plant derived from a single cell in culture seems only a question of time.

Some applications of this basic "nurse" technique are obvious for the study of pathological growths, of normal tissues, of early stages in embryological development, and so on.

Dr. White: The technique has been available for only a year or two.

Dr. Riker: That is right. We reported it in July, 1956, at the meeting in Briançon, and it appeared at the same time in *Science*, you may recall.

Dr. Skoog: What Dr. Steward says, obviously, must be correct. I don't claim that the solution of morphogenesis lies in any one particular substance. There is a tremendous number in the auxin category alone. Likewise there are many substances in the category of kinetin. We call them kinins, and we already have about 40 such compounds with similar physiological activity. These represent only two of the numerous categories of chemicals that must take part. However, I would like to make an analogy with sound and music. Only a few notes are required to get all kinds of sound including good music and bad. I believe that variation in only a few growth factors is enough to allow for the most widely different morphological end results. But far be it from me to say that the key to the solution of morphogenesis lies in any one particular chemical substance.

Morphogenesis in Animal Tissue Cultures[1, 2]

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The amount of work on morphogenesis in animal tissue cultures has become so great that it seems impossible and, obviously, impracticable to try to review, in a half hour even the work of the past 10 years. Fortunately, however, from time to time, review articles have appeared and presumably will appear in the future. In this connection I might mention chapter 10 of Geoffrey Bourne's *Cytology and Cell Physiology*, written by Miss Fell in 1950; a review entitled "Recent Advances in Organ Culture" by the same author published in *Science Progress* in 1953; and Borghese's article published by the Italian Anatomical Society in 1954, entitled "The Development of Isolated Embryonic Organs." Unfortunately for many of us, this excellent review is written in Italian.

According to the intention of this Conference I will try to present a framework for the discussion and use part of our own work to illustrate some of the principles. It will be impossible to cover all the important questions that might be suitable for discussion and therefore I have selected four points. There is, however, a further limitation of my talk: Morphogenesis is a term that, of course, covers the development of all morphological criteria from the molecular and submicroscopic levels to the microscopic and anatomical ones. So far, most of the work published in our field has been in the microscopic field and, consequently, I have decided to confine myself to that part only. But let us start and try to explore the field.

With classical methods of tissue culture (Harrison, Carrel, Ebeling, Fischer, and others) it soon became evident that only tissues with a relatively high regenerative capacity could be successfully cultivated. Consequently, mainly tissues with so-called vegetative intermitotic and perhaps some reverting postmitotic cell types (Cowdry) were used for the purpose. Therefore morphogenetic phenomena were bound to be rather rare. Moreover, differentiating and already differentiated tissues that were present at the beginning of the cultivation period, such as bone, muscle, and glandular tissue, either were eliminated by dissection or grad-

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[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8–12, 1956.

ually disappeared by degeneration, disorganization, or perhaps by dedifferentiation. At any rate, only colonies of intensively proliferating spindle-shaped, epithelial, or ameboid cell types remained after prolonged cultivation. Albert Fischer concluded from these facts that the high rate of proliferation in the tissues was the *cause* of the lack of morphogenetic phenomena. Champy expressed himself later as follows: "Morphological differentiation is a function of the speed with which cells divide mitotically," which seems to be a more careful expression than the one used by Fischer.

Not only in the *early* tissue-culture period but also in more recent times, results were obtained with improved techniques pointing toward the critical importance of avoiding extensive outgrowth if morphogenetic processes are desired. In fact, many solutions have been found leading to a suppression of an extensive outgrowth, such as changing the composition of the medium in such a way that growth-stimulating substances diminish or are left out (Fischer), changing the consistency of the coagulum either toward a very stiff or toward a very weak condition (Fell), moving the explant frequently from one place to another—as along a glass rod (Martinovitch), creating a liquid pool around the explant in the coagulum by making use of the fibrinolytic activity of the epithelium in culturing glandular tissues, or using fluid media, and many others. In fact, different types of tissue often have to be treated in a special way. Miss Fell mentions, for instance, in one of her reviews that avian limb rudiments "prefer" a stiff coagulum, while mammalian tissues, and according to our experience especially human endocrine tissues, on the contrary "prefer" a very weak coagulum, which becomes partly liquified by the fibrinolytic activity of the cells.

There is little doubt that as a rule extensive proliferation and morphogenesis do not go hand in hand and one might conclude that at least for the vegetative intermitotic cell types active proliferation "hampers" the appearance of morphogenetic processes. However, I do not like to go so far as to state that a *lack* of proliferative activity promotes or is the cause of such processes. May I cite the result of one of our own experiments?

Strain cultures of periosteal "fibroblasts" from a 15-day-old chicken embryo were cultivated in Carrel flasks in a coagulum comprising rooster plasma (8 parts), Ringer's solution (10 parts), and a press juice of 7-day-old chicken embryos (2 parts). After coagulation, the flasks were thoroughly washed with Ringer's solution and then both control and experimental cultures received a fluid phase of 12 parts of a 20 percent press juice of 7-day-old embryos in Ringer. This fluid phase was refreshed every 2nd day; the control cultures each time received the same kind of medium; experimental cultures received, after 2 days, a press juice of 10-day-old embryos instead of 7-day-old embryos, after 4 days, 1 of 14-day-old embryos, after 6 days, 1 of 18-day-old embryos, after 8 days, serum of 2-day-old chickens and, after 10 days, serum of an adult chicken (this is what we have called an ascending range of body fluids). In all flasks (experimental and control) active proliferation practically came to a

standstill after 4 to 5 days of cultivation, and after 6 days hardly any mitotic cell division could be found. Nevertheless, in the majority of cases the formation of a prebone substance and eventually of bone occurred only in the experimental cultures, while in the control cultures no morphogenetic phenomena whatsoever were present. Consequently, at least for this process, a lack of proliferation was certainly not the cause but probably a condition for the histological differentiation processes.

As my second point I would like to mention the role of the culture medium, including the significance of special compounds such as hormones and vitamins. The results of the experiments just described indicate that not only in very early embryonic development but also in later embryological stages, "outside" factors can be decisive for the realization of developmental processes in tissues that have already undergone an adequate determination at an earlier period. The question arose whether also in the adult tissues "outside" factors can be of importance for the maintenance of specific structures. In this respect, I may mention that explanted fragments of the fully differentiated anterior pituitary of 3-month-old rabbits do not even survive in a medium containing fibrinogen and a press juice from young embryos. Chick-embryo fibroblasts, however, do survive in such a medium. In contrast, explants cultured in blood plasma instead of embryo press juice show some outgrowth, an encapsulation of the whole fragment, and in the interior the preservation of different specific types of cell. I do not claim this to be a general rule, but I would like to present for discussion the necessity of providing, at least for more advanced embryonic tissues, not only adequate basic nutrients but also more or less specific factors governing the realization of morphogenetic processes. I imagine Dr. Fell and Dr. Wolff also will contribute on this particular point.

As far as hormones and vitamins are concerned, a great deal of work has already been done. Miss Fell described what may be called a modulative effect of large doses of vitamin A on explants of the chick skin. Under the influence of this compound a transformation of the keratinizing epidermis toward a mucin-secreting epithelium, occasionally with ciliary cells, occurred. Weiss and James were able to produce the same kind of effect after only a short exposure of skin-cell emulsions to vitamin A. Wolff studied the feminizing effect of ovarian hormones on explants of the early avian testis. Schaberg, of our institute, recently found an extensive formation of cells that resembled those of the fasciculata and that were in explants of the glomerular zone of the adrenal cortex from 5-day-old rats under the influence of ACTH or of fresh anterior-pituitary tissue. However, combined cultivation with anterior-pituitary fragments that had been precultivated for about 1 week prior to the confrontation resulted in no effect whatsoever. Nevertheless, the cells of these anterior-pituitary explants were still capable of releasing an ACTH-like factor, because after addition of a tissue fragment of the hypothalamus (eminentia media and its surroundings) the effect could be re-established. Estimations of the ketosteroids in the culture medium as a measure for the func-

tion of the adrenocortical cells pointed in the same direction. These are a few examples, but I trust that they will suffice to stimulate the discussion on the significance of hormones and vitamins on morphogenetic processes.

The third point, which has already been touched upon, is the significance of intercellular relationships for the realization of morphogenetic processes. It was Champy who stated that epithelial cells hold their degree of differentiation only if connective tissue is present. Albert Fischer published experiments on the combined cultivation of strain cultures of heart fibroblasts and iris-pigment epithelium and observed tubule or cystlike formations in the centers of the explants. Also, in the migratory zones of strain cultures certain typical arrangements of epithelial cells occurred as soon as a contact became established with spindle-shaped elements. In these cases there is often found a layer of fibroblasts arranged parallel to the borderline between the 2 layers and perpendicular to the epithelial cells. This indicates that the mere contact of these tissues led to other arrangements of cells.

Thomson described how enamel cells function in odontoblasts in culturing tooth primordia. Recently Grobstein studied the significance of mesenchymal elements for the development of organotypical glandular structures of the salivary gland. He was able to show the specific role of the mesenchymal elements of the gland itself. Only in direct contact with salivary-gland mesenchyme did the glandular primordium develop in an organotypical way. He stated that this typical effect lessened and finally disappeared when the mesenchyme elements were cultivated as separate cultures for some time prior to combining the glandular epithelium and the mesenchyme *in vitro*. If I interpret his results correctly, the continual presence of the mesenchymal elements is of importance in the development and maintenance of the typical glandular structure. This kind of effect is often referred to as an "induction" phenomenon. I do not feel quite justified in using this term in this connection because, in my opinion, it has a slightly different meaning in experimental embryology. In this discipline induction does not merely mean a contact action but also a specific reaction in a limited area, with a definite reaction pattern. Moreover, the duration of the interaction is only necessary for a limited period of time and during a sensitive period of the tissue to be influenced. After the determination has taken place the contact is no longer essential and, consequently, the maintenance of the structure does not depend on continuous contact with the "inducing agent."

I agree, however, that in later developmental stages, and perhaps even in adults, continuous interactions between cell types seem to be of great importance for the maintenance of typical structures. In some of our own experiments fragments of the anterior lobe of the 3-month-old rabbit pituitary were cultivated in close contact with posterior-lobe tissue. Only along the borderline and only as long as the contact remained intact did an intermediate-lobelike structure develop at the site of the contact with the anterior-lobe explant.

May I refer also to the interesting studies of reaggregation of tissue elements after separation of the cells, for instance by trypsin, as described by Moscona and by Weiss? During cultivation, such cell suspensions (*e.g.*, of the pituitary primordium) show a reaggregation in a rather mysterious way and organotypical development may take place *in vitro*. We might, perhaps, discuss whether comparable aggregation phenomena may not also function in normal embryogenesis.

Finally, I would like to mention the possible significance of the developmental stage of the organs or organ fragments to be cultivated. I think one can say that as a rule the early embryonic primordia are, relatively, the easiest tissues to cultivate. Moreover, partly because of the relative speed of the developmental processes at this time, they already show rather extensive and "normal" morphogenetic phenomena during a short cultivation period. This statement does not mean that the best morphogenetic phenomena will *always* be obtained with the youngest possible primordia. The region must first be capable of a certain degree of self-differentiation. It has been demonstrated by Miss Fell that certain regions of mesoderm beneath the buccal epithelium of the branchial arch of the chick embryo have acquired the ability to form cartilage by the 3rd day of embryonic life and not earlier, while osteogenic cells destined to form certain parts of the mandible arise at the proximolateral end of the mandible only at the 4th day of development. Wolff described the behavior of the gonads of the mouse embryo and found that gonads from 10- to 11-day-old embryos developed only sterile structures, while the rudiments taken from 12- to 13-day-old embryos developed into complete gonads with oocytes. Not only must the tissue have become capable of developing in a certain direction but also in later stages of development there often seem to be differences in what one might call explantability. Such differences perhaps coincide with special nutritional or other metabolic needs. Thus, thyroid tissue is easier to cultivate if follicle formation has not yet begun. From the adrenal cortex the glomerulosa cells are far easier to explant than the fasciculata cells, though such cells can be formed from glomerulosa cells during cultivation. Further, the cranial part of the human fetal ovary is extremely difficult to cultivate as "organ culture." However, the caudal part easily adapts and gives rise to the formation of cordlike proliferations with numerous newly formed oocytes.

Difficulties in the proportional development of explanted primordia sometimes occur if some particular kind of cooperation between different cell types cannot be created. Miss Zaaijer in my laboratory observed that human fetal phalanges during a well-defined period of their development could develop normally *in vitro* only if an encapsulation by epithelial elements was created. In this case we suggest that the epithelial cover acted primarily as an actively selecting membrane and thus created the local climate that was necessary for a proportional growth development. Moreover, as soon as epithelial encapsulation of a tissue fragment has occurred, the explants appear to become less dainty as to the actual composition of the culture medium.

All this tends to indicate that organotypical development is favored as soon as an explant can behave as a "unit," as some kind of "closed system." This also would be in agreement with the experience that small-sized primordia are easier to cultivate than fragments of larger organs, which do not always succeed in developing such operational units by means of regeneration or reorganization. I would like to close with the remark that fragments of the cortex of the human fetal ovary in which complete encapsulation has occurred go on to a reorganization that eventually leads to the formation of a miniature ovary with oocytes in the meiotic prophase.

DISCUSSION

Dr. Wolff: I agree with Dr. Gaillard's report on the conditions of organized cultures. Not having time to discuss the whole problem, I should like to stress intrinsic factors. Among inside factors there are two important phenomena: encapsulation, which Dr. Gaillard mentioned at the end of his report, and tissue relationships.

1) *Encapsulation.*—Formation of a limiting membrane is one of the most important conditions for the success of organ cultures. Numerous examples can be cited from our own experiments. Mrs. Haffen and I have perfected a method that gives pure organotypic cultures, both in birds and mammals, in all cases that we have tested. Almost all organs (liver, testes, ovary, syrinx, mesonephros) were able to develop an encapsulating membrane under the conditions of our technique. This membrane is not necessarily similar to the normal capsule of the explanted organ. It may be formed by any kind of cells that happen to be at the external surface when they are explanted. If a mouse testis of a 16-day-old embryo is minced and the small pieces reassociated on our standard medium, germ cells of the testicular tube may form the outside wall of the explant. The spermatogonia then reorganize in a kind of keratinized epithelium, the most external cells becoming flat and dehydrated, the internal ones resembling the polygonal cells of a true multilayered epithelium (fig. 1, plate 48). When small pieces of liver are reassociated, the external capsule may be formed by connective tissue or by true hepatic cells. Both cases may appear in the same explant (fig. 2, plate 48). Numerous examples of such processes could be mentioned. Organs that are deprived of their normal capsules tend naturally to rebuild their capsule with any tissues available.

2) *Interactions between tissues.*—Among other factors that maintain the cohesion of structures and the unity of organs in culture are the interactions between cells. Such interactions may be attractions, repulsions, or stimulations to growth or differentiation. Borghese, Moscona, and Grobstein have done excellent work in this field. I must refer also to the recent research by Holtfreter and Townes on amphibian embryos. Gastrulas and neurulas that have disaggregated into suspensions of separate and intermingled cells reorganize into almost normal embryos when they are replaced in normal conditions—as if cells of the same origin could recognize one another. This conspicuous phenomenon is due to special relationships between cells, especially to attractions and repulsions between certain types of cells. We may identify results of this type in our own experiments. They are particularly striking because they are concerned with heterogeneous associations between two species, mouse and chick embryos. For example, when lungs of the two species are associated, bronchial buds of the mouse lung develop and proliferate in the middle of chick pulmonary mesenchyme (fig. 3, plate 48). Chimeric associations of testis and lung show a collaboration of the epithelial tubules of the two species. In a chimeric testis, seminal tubes are reorganized whose walls are composed of alternated mouse and chick cells (fig. 4, plate 48). Spermatogenia of both species coexist in the middle of these mixed tubules. The same phenonenon is observed in explants in which bronchial epithelia from chick and mouse fuse into chimeric bronchiae.

Finally, I should like to mention associations between liver and other organs. Liver cells move and creep on the external surface of the forming organ. Several of them ultimately invade all the spaces inside the organ. In other parabioses, mouse fibroblasts migrate far from their origin into the tissues of the chick, following determined pathways (for example under the basal membrane of epithelium). Plate 48, figure 5 shows a combination of mouse and chick ovaries in which the fibroblasts are migrating along the basal membrane.

PLATE 48

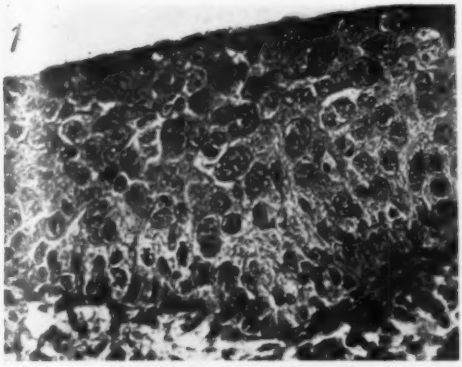
FIGURE 1.—Epithelial wall, formed by the spermatogonia, when they are at the periphery of an explant. × 400

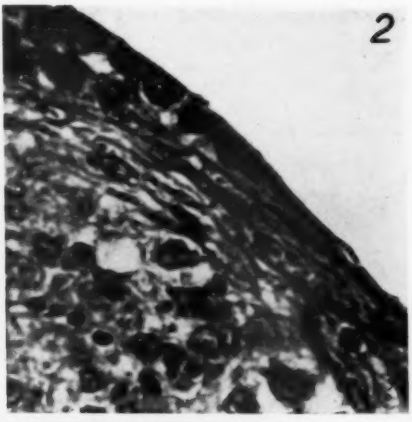
FIGURE 2.—External membrane of a piece of liver, cultivated *in vitro*, formed partly by hepatic cells (*left*) and partly by connective tissue (*right*). × 730

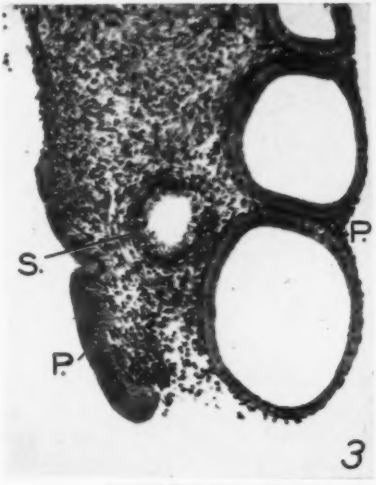
FIGURE 3.—Epithelial tubule of mouse lung growing in the mesenchyme of chick lung: P—chick bronchiae; S—mouse pulmonary tubule. × 180

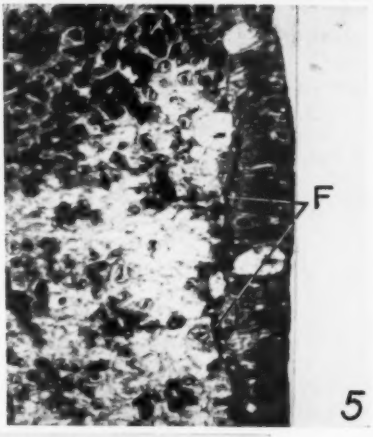
FIGURE 4.—Association between duck and mouse embryonic testis: C—duck testis; S—mouse testis. *Arrow* indicates region in which a chimeric seminal tube was built. × 120

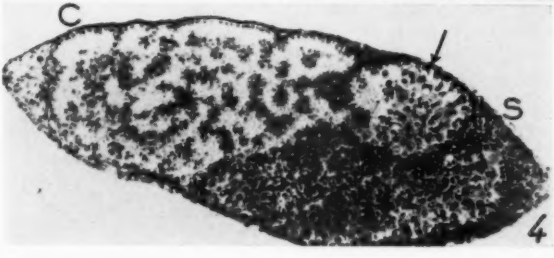
FIGURE 5.—Association between a mouse ovary and a duck testis. Fibroblasts (F) have migrated from the mouse explant along the basal membrane of the germinative epithelium of the duck testis. × 300











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In conclusion I can say that such xenoplastic associations, although, of course, unusual, give evidence of 3 types of attractions which operate in organotypic cultures: 1) collaboration between homologous types of structures; 2) attraction between different types of cells, such as epithelia and mesenchyme; 3) attractions and migrations of cells in a heterogeneous living medium. Such interrelations reveal some factors that are responsible at the same time for organ unity and for organized culture.

Dr. Grobstein: Dr. Gaillard called our attention to the problem of tissue interrelationships in development, and Dr. Wolff has just given some beautiful examples. We recently have been studying a case of this kind *in vitro*, and I should like to take a few minutes to bring you up to date on what we know about it. The facts may have relevance not only for our subject today but for our discussions yesterday on nutritional and other requirements for maintenance of the differentiated state.

First, may I say a word on the question of the use of the term "induction." Dr. Gaillard has mentioned his feeling that "induction" is perhaps not suitably applied to the interrelationships between epithelium and mesenchyme that seem important in the development of a number of vertebrate organs. Whether or not to stretch the term to cover these phenomena is, I think, largely a matter of taste at the moment. The fact is that there is no complete agreement among embryologists as to how to limit the term and certainly no agreement as to the nature of the mechanisms involved. I personally am inclined to feel that we are dealing with a spectrum of processes with several common characteristics, but with important differences as well. All represent developmentally significant direct tissue interaction, which seems to me the crux of what we can now mean by induction. Examples range from the early primary organizer effect—with all of its complexities—through such relatively simple interactions as we have been describing in culture. The term "induction" can be conveniently applied to all of them to emphasize their similarities but without assuming that they are identical, particularly with respect to mechanism. It remains for the future, as our knowledge increases, to tell us where to draw the line between the several types of developmental interaction which may exist and to define "induction" more positively.

In any event, the case that I will now take up is generally accepted as induction—the formation of epithelial tubules in nephrogenic mesenchyme. Metanephrogenic mesenchyme from an 11-day-old mouse embryo will produce these tubules *in vivo* and *in vitro*, providing it is in association with the ureteric bud. Deprived of this association, such mesenchyme fails to produce tubules in culture, but it can be induced to do so by clustering fragments around a piece of embryonic dorsal spinal cord. The differentiation of metanephrogenic mesenchyme in culture thus is controlled by some influence exerted by the ureteric bud or the artificially supplied spinal cord. We have been attempting to obtain additional information on the nature of this influence. From the point of view of the embryologist, this is a study of the mechanism of an inductive process; from the point of view of the tissue culturist, we are asking what must be supplied to initiate a particular differentiation *in vitro*—with the clue that the something can be supplied by living embryonic spinal cord.

In approaching this problem we were fortunate in finding that the reaction can take place across thin molecular or membrane filters that definitely exclude penetration by whole cells (*1*). The question was left open as to the extent and significance of penetration of the filter by fine cytoplasmic processes that might make contact in the depths of the filter. The phase-contrast microscope showed abundant material of uncertain nature in sections of the more porous (TA) of 2 filter types used, sparse and shallow penetration in the less porous (TH) of the 2 types. We have recently examined still less porous (TV) filters, which pass the inductive activity in reduced degree, and could see no penetration with the phase-contrast microscope. In collaboration with Dr. A. J. Dalton, we now have studied with the electron microscope (*2*) all 3 filter types, fixed while separating tissues in inductive interaction. The observations are in accord with the earlier ones with the phase microscope as to the extent of penetration of the 3 filter types, and demonstrate that the previously observed penetration of TA and TH filters is at least largely cytoplasmic. Electron micrographs of sections show that

cytoplasm is clearly recognizable when it does penetrate these more porous filter types, and is virtually completely excluded from the least porous TV type. From these observations the conclusion seems safe that this particular inductive interaction can occur despite a cytoplasmic separation of about 20μ.

Further information on the nature of the influence comes from the following type of experiment. The reaction will proceed across 3 layers of the TH or TA filters, each layer being approximately 20μ thick. The middle filter layer can be replaced by a cellophane disc perforated by a small hole (0.15 mm.), and the interacting tissues on either side arranged so that the hole is included between them. Tubules now appear in the mesenchyme only over or in the immediate vicinity of the hole; everywhere else the cellophane blocks passage of the inductive influence. It would appear, therefore, that whatever the nature of the influence, it is noncellophane-passing, but can traverse the fluid-filled space of 20μ thickness, represented by the hole in the cellophane.

From these observations, and others, we are led to suspect and examine the possibility that large molecular materials, normally closely associated with cytoplasm but experimentally separable from it to some degree, are involved in inductive processes. As a corollary, the possibility is strongly raised that such materials may be of importance in initiation (and maintenance?) of the differentiated state *in vitro*.

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Dr. Fell: I should like to say something about Dr. Gaillard's first point, the relationship between proliferative activity and differentiation in cultures. This has been a very controversial subject for a long time and I think there is some confusion of thought involved. It may be quite true that very violent mitotic activity is antagonistic to differentiation. But I can only say that my healthiest, longest-surviving, and best-differentiated cultures always have plenty of mitoses in them—usually the more mitoses the better the culture and the better the differentiation. In some recent experiments that I have been doing on the metaplastic action of vitamin A on the scaly shank skin of rather late (13-day-old) chick embryos, I have even seen plentiful mitoses in secretory cells that had already begun to form mucin; this seems to me a clear indication that we must not generalize about this question of mitosis being always, in all tissues, antagonistic to differentiation.

I think that much of the confusion arises from the fact that in the conventional types of tissue culture, conditions which favor very active mitosis also favor very profuse cell migration. The great enemy of the organ culturist is not so much mitosis as emigration; sometimes measures which restrict both mitosis and emigration are used and encourage differentiation, not because they reduce mitosis, but because they restrict emigration. There is, however, a purely practical reason why mitosis should not be too active in an organ culture—we do not want the explant to become too large. The reasons for this are quite obvious, because *in vitro* the tissue has no circulation and has to feed, respire, and excrete exclusively through its outer surface. If we stimulate the tissue to grow too much, then the interior will become necrotic; this will probably have a toxic effect on the more superficial cells and the culture will die for that reason. Ideally, what we organ-culture people like is to encourage our explant to reach an optimal size and then to produce as much mitosis as will compensate for cell death in the explant.

I should just like to refer briefly to this question of the importance of what Dr. Gaillard has called "outside factors" in the cultivation of differentiating tissues. This can be an extremely exasperating business, as anyone knows who has tried to

grow differentiated tissues, because so often we are completely in the dark about what is lacking in our medium or even what is in excess, and we just have to try to modify it in a purely empirical and very unscientific way. Sometimes, however, we can be a little more rational in our approach; my colleague, Dr. Lasnitzki, has a very nice example of the importance of androgen in maintaining the normal structure in the young prostate gland of mice in organ cultures. It is this question of the outside factors that is going to be a considerable problem in growing organ cultures in synthetic medium. Some organ cultures do quite well and differentiate very favorably in synthetic media but others fail to form certain constituents; I think we may find that it is easier to grow unorganized tissues in artificial nutrients than to obtain a given type of differentiation in artificial media, and probably we shall find that we have to tailor the medium to each type of organized tissue that we want to grow.

Dr. Moscona: The problems discussed here so far related mainly to morphogenetic phenomena on the tissue level, that is, to interactions between two organs, organ rudiments, fragments of tissue, or between tissues and chemical activators. I should like to discuss cellular interactions and some of the problems and possibilities arising from the study of morphogenetic phenomena on the cellular level. What is the relationship between the cellular pattern and architecture of embryonic tissue and its subsequent differentiation? This type of problem can be approached experimentally by destroying the integrity of the tissue without affecting the viability of the cells and by examining the ability of such cells, removed from their original interrelationships, to reconstruct a tissue fabric suitable for further development. The disruption of the cellular pattern of a tissue may be either partial or complete, *i.e.*, resulting in a total dissociation of the tissue into discrete, individual cells. It has been found that, under appropriate conditions, such completely dissociated cells aggregate into clumps, re-establish tissuelike association, and subsequently differentiate histotypically in accordance with their origin (J. Anat. 86: 287, 1952). Aggregates of chondroblasts develop into cartilage, those of nephroblasts form nephric tubules, retinal cells give rise to retinal rosettes, and so on.

This quite remarkable capacity of dissociated cells to re-establish contiguity *in vitro* and to reconstruct tissue fabrics offered various intriguing experimental possibilities; it also raised numerous intricate questions, like the one mentioned by Dr. Gaillard, concerning the mechanism of the "mysterious process of aggregation." Another no less interesting question was: What goes on inside a cell aggregate, and especially in aggregates composed of more than one type of cell, in the course of association and organization? Do cells of different histogenetic types become intermingled and distributed at random in composite aggregates, or do they become sorted out into groups according to type? Is the cellular pattern of a reconstructed tissue affected by the intermixture with cells of a different type? Figure 1, plate 49, shows a suspension of chondrogenic cells from the limb bud of a 4-day-old chick embryo, prepared according to the trypsin-dissociation procedure developed at the Strangeways Research Laboratory (Exper. Cell Res. 3: 535, 1952). When plated out on a plasma clot, such discrete cells spread out to form a typical fibroblastic culture. If, however, the cells are suspended in a suitable liquid medium, they remain rounded, migrate readily, and soon begin to cluster into groups that eventually become compact aggregates (figs. 2, 3, plate 49). As they reach macroscopic size, the aggregates can be harvested and transferred for further cultivation on a clot. The aggregation takes place in a hollow-ground slide in a medium consisting of chick serum, embryo extract, and physiological salt solution. I may say, parenthetically, that changes in the composition of the culture medium may markedly affect this process; it is prevented, for instance, when a small quantity of horse serum or some human sera is added to the medium, which then affects chick cells so that instead of aggregating they spread out on the glass and form sheets.

The shape of aggregates varies according to the type of cells involved; one is tempted to draw an analogy with variations in the shape of crystals of different chemical composition. Chondrogenic-cell aggregates are usually spherical, mesonephric aggregates have a corrugated appearance, retinal cells form elongated and forked aggre-

gates. When two different types of cells are intermixed, the shape of the resulting aggregates varies according to the identities of their constitutent cells. Thus, composite aggregates consisting of retinal plus mesonephric cells have a branched, mycelium-like form. As stated before, aggregates consisting predominantly of one type of cells become organized in a pattern characteristic of the particular type of cells involved; for example, embryonic retinal cells reassociate to form concentric rosettes in which the cells are orientated towards a central lumen. In the presence of cells of another type, for instance mesonephric cells, a different pattern of association may arise. In the early phases of aggregation both types of cells are found to be dispersed at random throughout the cluster; neither the darkly nucleated retinal cells nor the nephric cells have as yet formed any recognizable structures. Subsequently, the aggregates undergo a structural organization resulting in the two types of cells becoming grouped in different regions, according to their identities. This regionalization is concurrent with, or followed by, histodifferentiation into retinal and nephric structures. In this case, however, the retinal cells, in addition to forming rosettes, become frequently arranged around nephric tubules as convex plaques of retinal epithelium, with the sensory surface of the very elongated cells exposed to the culture medium. Both the shape of the retinal cells and their characteristic histogenetic pattern may thus become modified in a heterotypic association.

Another example of a composite, heterotypic aggregate is seen in figure 4, plate 49, which shows a structure formed by intermixed chondrogenic and nephrogenic cells. In spite of their initial intermingling, cells of both types eventually reconstituted their respective tissues: nephroblasts became organized into tubules, chondroblasts grouped to form cartilage. Evidently, the cells retained in this situation their type specificities and their characteristic histogenetic properties.

I should like now to mention two of the problems that these composite aggregates present. First, the manifestation of histogenetic activity in heterotypic aggregates may be profoundly affected by the relative proportions of the diverse cells; the mere presence of cells of a certain type in a heterotypic system does not insure the reconstitution of the respective type of tissue. Unless present above liminal proportionate concentration, which varies according to cell type, stage of development, and combination, the cells (or their products) become "diluted" in the system and do not give rise to a coherent tissue fabric (Proc. Soc. Exper. Biol. & Med. 92: 410, 1956).

The other problem is that of identification of the individual cells in heterotypic combinations. To follow the fate of the intermingled cells in a composite aggregate so as to determine their ability for retention of their original properties, one must be able to clearly identify the cells and to distinguish one type from another. Various marking procedures were considered and rejected, and, eventually, upon the suggestion of Dr. Wolff's work, the marked morphological differences between chick and mouse cells were exploited for this purpose. A procedure was worked out, whereby embryonic chick and mouse cells intermixed in suspension in suitable proportions, produced aggregates in which cells from both animals became incorporated. Such aggregates differentiated into chimaeric chick-mouse tissues (Proc. Nat. Acad. Sc. 43: 184, 1957). The generic and the histotypic identity of the individual cells could be readily established: in preparations stained with hematoxylin and Biebrich's scarlet, mouse-cell nuclei stained a deep blue while chick-cell nuclei were pink. A variety of isotypic and heterotypic combinations of chick and mouse cells was studied and 2 examples will be mentioned here. Figure 5, plate 49, shows an example of an isotypic combination, *i.e.*, of an aggregate of chondroblasts from both species: The cartilage thus produced is chimaeric, consisting of completely interspersed chick and mouse cells. The cells of both species are embedded in a common matrix and form a continuous fabric. The results were quite different, however, if 2 histogenetically different types of cells were intermixed, for instance, chick nephroblasts and mouse chondroblasts (fig. 6, plate 49). In such heterotypic aggregates the cells were found to have formed separate groupings according to type: chick nephroblasts formed tubules, mouse chondroblasts formed cartilage. There were no chick cells in the cartilage and no mouse cells in the tubules. Essentially similar results were observed in other iso-

and heterotypic combinations of chick and mouse cells. It was thus concluded that, under the conditions examined, the dissociated and aggregated cells retained their original identities; that they formed preferentially type-specific groupings, regardless of the generic origin of the cells and reconstituted tissue fabrics in accordance with their pre-established properties.

The tenacity of these cellular faculties was, perhaps, even more emphatically demonstrated when tumor cells were combined with embryonic cells. In the one case to be mentioned here, dissociated cells of pigmented melanoma S91 of the mouse were intermingled with hepatic cells from a 6-day-old chick embryo. Aggregates were formed in which the hepatic cells became consistently grouped on the surface in the form of hepatic parenchyma surrounding a core of the melanotic tissue (fig. 7, plate 49).

PLATE 49

FIGURE 1.—Smear of a cell suspension of dissociated chondrogenic cells from the limb buds of a 4-day-old chick embryo. Hematoxylin and eosin. × 500

FIGURE 2.—Twenty-four-hour aggregates of chondrogenic cells, in culture. × 30

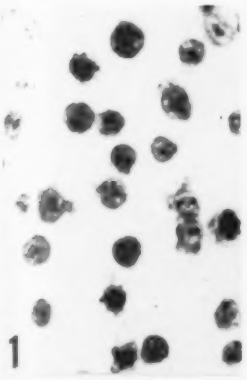
FIGURE 3.—Section through similar aggregates as in figure 2, after 48 hours' cultivation in liquid medium; beginning of chondrification. Hematoxylin and eosin. × 80

FIGURE 4.—Section through aggregate formed in suspension of intermingled mesonephric and chondrogenic cells from a 4-day-old chick embryo, after 7 days in culture. Hematoxylin and eosin. × 120

FIGURE 5.—Section through aggregate formed in suspension of intermingled mouse and chick chondrogenic cells, showing the chimaeric structure of the cartilage, 3-day culture. Hematoxylin and Biebrich's scarlet. × 280

FIGURE 6.—Section through aggregate formed in a suspension of mouse chondrogenic and chick mesonephric cells, 3-day culture. Hematoxylin and Biebrich's scarlet. × 220

FIGURE 7.—Part of a section through aggregate of hepatic cells from a 6-day-old chick embryo and mouse pigmented melanoma (S91) cells, 48-hour culture. Hematoxylin and Biebrich's scarlet. × 420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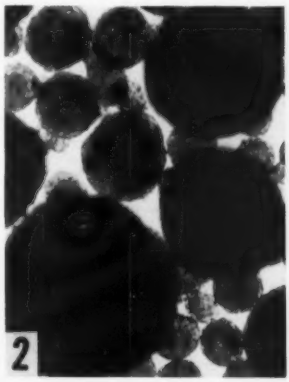
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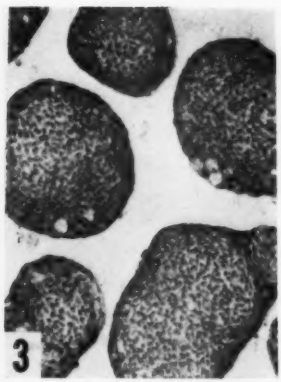
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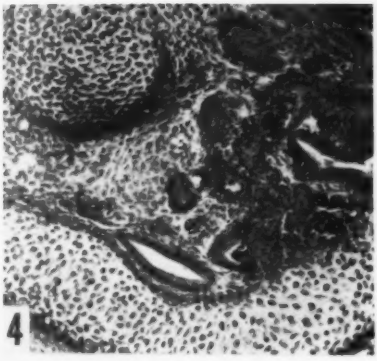
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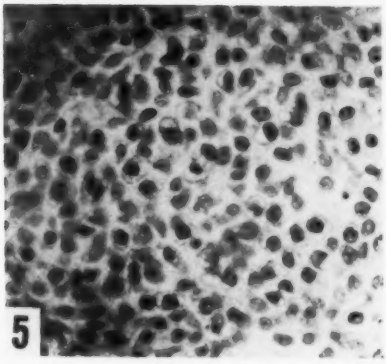
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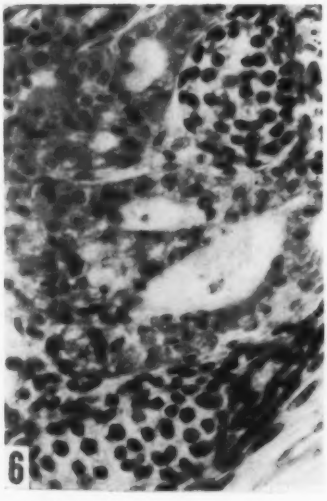
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Dr. Earle: From these cultures, particularly as demonstrated by Dr. Moscona, I was impressed by the similarity of certain structures to some which we have seen, in growing cells in fluid-suspension cultures, subjected to rapid agitation. One of the cells that we studied was the HeLa strain of a human squamous-cell carcinoma from the cervix of the uterus. Even at an agitation of 13,000 revolutions per hour, this cell on proliferation consistently produces many very beautiful little spherical masses of cells, up to half a millimeter in diameter. In our laboratory we have quite casually called these "gemmules." Their production is extremely characteristic of this cell strain. It is of interest that this cell type does have the tendency to produce these gemmule-like structures even under such rapid agitation.

Under many of our presently used experimental conditions, proliferation and recognizable differentiation do not both occur at maximum levels. However, I agree with Dr. Fell, that evidence now available does not justify the conclusion that proliferation and differentiation are antagonistic. I wonder whether more extended study will show that further modification of the culture medium or introduction of other change in experimental conditions may ultimately make possible rapid cell proliferation associated with at least some types of rapid and progressive morphologic and/or functional cellular differentiation.

Dr. Gaillard: As far as the problem of inhibition of mitosis and differentiation is concerned, I think that we ought to keep in mind that the organism consists of different types of cells. We make, for instance, a division between vegetative intermitotic cells and differentiating intermitotic cells. As regards the differentiating type, and considering the organism as a whole, there is plenty of evidence that mitosis and differentiation can occur simultaneously in one and the same organ. However, the presence of mitotic divisions does not necessarily mean that the cells that are in the process of division show or actually take part in a differentiation procedure. Apart from the dividing elements there are always plenty of nondividing cells present that could be responsible for the differentiation. I did not mean to say that there are no mitotic divisions in cultures showing differentiation phenomena, but that there seems to be evidence of an incompatibility between the process of division and the process of differentiation, if one considers one and the same cell.

Apart from this I agree with Dr. Fell that migration can interfere with organization because migration of cells in a culture leads toward disorganization of the tissue.

Dr. Murray: I was going to elaborate a little on one of Dr. Gaillard's points. In this connection, we heard Dr. Fell say, near the end of her discussion, that in an organ culture a mitotic rate that just about keeps pace with necrosis results in a desirable situation. This, of course, is quantitatively totally different from the division rate in Dr. Earle's cultures. If you have a cell whose intermitotic period is 8, 16, or even 20 hours, it is expecting too much of this cell to form differentiated structures, in between the very extreme physical and chemical changes that it undergoes in mitosis. It would be expecting too much of a skeletal-muscle cell, for example, which takes days to form its oriented micelles, to carry on mitosis and differentiation at the same time.

Dr. Martinovitch: I should like to comment in regard to the necessity of a capsule for the preservation of the integrity of a highly organized piece of tissue *in vitro*. We decapsulated infantile rat adrenals and kept them *in vitro* for as long as 2 months. Cortical cells on the surface gradually died out, but medulla, or a good portion of it, survived for 2 months and when subsequently transplanted lived happily ever after.

Dr. Lasnitzki: I would like to comment on one point relating to outside factors mentioned by Dr. Gaillard. We found that in organ cultures of the mouse prostate the response to hormones depended on the age and sexual maturity of the animal from which the organ was derived.

In cultures derived from 4- to 6-week-old mice, dedifferentiation takes place *in vitro*: the lining epithelium of the alveoli is reduced in height and the folding lost, while the interalveolar connective tissue is increased. On the other hand, in explants obtained from sexually mature mice, the structural differentiation is maintained even after prolonged periods of cultivation.

Addition of testosterone propionate to the medium in a concentration of 25 μg. per

ml. preserves the differentiation in explants of young prostates but causes extensive hyperplasia of the alveolar epithelium in sexually mature glands.

This result indicates that the young, sexually immature glands depend on the continuous supply of androgenic hormones, which is not available in sufficient quantities in the culture medium, while the older glands are either independent of it or need smaller concentrations to maintain differentiation. The induction of hyperplasia in the sexually mature glands suggests that the epithelium has not become refractory but rather more sensitive to the hormone.

Dr. Wilde: I think that one should consider necrosis and necrobiosis on the cellular level as a possible morphogenetic mechanism. There are certain new data in chick embryology in which the selective or positional death of cells seems to bear a distinct relationship to the subsequent differentiation of an organ. I refer to the work of Saunders (*1*, *2*) in which the chick limb bud shows a particular area of necrosis which aids in the modeling of the upper limb. To supplement Dr. Fell's comment, there can be a positive morphogenetic aspect to necrosis.

I think the contrast between mitosis and cellular differentiation comes from observations on mitosis in living cells. One sees a withdrawal of cell processes—a rounding and apparent loss of structure within the cell. This is largely a question of what the eye sees and not necessarily of what the cell is doing. For instance, in certain well-differentiated pigment cells, where the pigment is in part already formed, it does not disappear as the cell goes through mitosis. The observer sees a pigmented cell throughout the process of mitosis. This would still be recognized by the morphologist as a differentiated cell, clearly distinct by reason of its pigment content. The argument can be established for other cell types.

References

(*1*) Saunders, J. W., Jr.: The origin of the definitive wing contour in the embryonic chick, with special reference to the shaping of the elbow. (Abstract.) Anat. Rec. 117: 559–560, 1953.

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Dr. Leighton: Dr. Moscona, you have discussed the reconstitution of tissue architecture by manipulating populations of cells and observing their tendency to form functionally organized aggregates of cells. Have you observed the multiplication of living units at the aggregate level? I have the impression from my experiments that duplication of living units not only occurs at subcellular and cellular levels, but that multicellular aggregates increase in number over a period of time by a kind of division of the aggregates. Have you made any observations that relate to this possibility?

Dr. Moscona: We have not noticed division of aggregates in our material.

Some Trends in Research on Cellular Differentiation and Morphogenesis [1,2]

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At a recent microbiological meeting a microbiologist, commenting on problems of cell differentiation and morphogenesis, stated that embryology ought ultimately to be studied in the embryo (*1*). It may be of interest to know what embryologists have to say on this matter. If we judge from the literature of these last years, we may conclude that they do not seem to share the opinion of the microbiologist. The tendency of embryologists is, in fact, more and more to shy away from the embryo and to concentrate their efforts instead on embryonic cells isolated *in vitro*. The reasons for such a shift are numerous. The *in vitro* method makes it possible: 1) to study living cells and confront them with a gamut of physical and chemical agents; 2) to make use of more precise methods of analysis in defined experimental conditions; 3) to replicate the experiments at will and evaluate the results on a statistical basis (an opportunity rarely, if ever, enjoyed by the experimental embryologist); 4) to control every step of the experiment and discontinue or modify it and thus avoid the waste of material and time which threatens all investigations in the living embryo.

Since very precise tools of analysis are now available to embryologists, it is understandable that many of them, dissatisfied with the old techniques, turned away from the embryo in favor of embryonic cells grown *in vitro*. This last decade witnesses such a trend and the promising results of this new approach to old problems.

One may now ask what penalties the embryologist has to pay when he decides to set aside the embryo in favor of shreds of embryonic tissue or of cellular groups torn away from the embryo by means of physical or chemical procedures. The most obvious one in the present instance is to lose sight of one of the most significant aspects of embryological development—the dynamics of morphogenetic processes and of cellular interactions that occur in the living organism but do not take place *in vitro*. Another disadvantage is the impossibility of studying cells in their normal environment. In searching for the best artificial media,

[1] Received for publication May 28, 1957.
[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8–12, 1956.

embryologists swing between two opposite poles: For some, the best medium is the one which reproduces with the highest fidelity the conditions encountered by the cells in the embryo, and, for this reason, they resort to media prepared with embryonic extract and coelomic fluid whenever possible; for others the best medium is the one in which all constituents are defined, and it should therefore be prepared with known chemical ingredients. The first tendency prevailed in the early 1920's, when the method of tissue culture was generally adopted by embryologists and cytologists, and lasted until the past decade; the second is favored at present as prerequisite for more rigorous conditions of experiment. Another reason for this recent trend toward better-defined and simple media may be found in the disillusionment that followed the first period. It soon became manifest to students of tissue cultures that no medium—no matter how closely resembling the fluid bathing the cells in the organism—provided suitable conditions for normal differentiation. The loss of morphological characteristics of different cell strains explanted *in vitro* with the hanging-drop technique came as a great disappointment to many histologists and shed doubt on the validity of the new method. The concept was developed that cells dedifferentiate *in vitro* and revert to a primitive, totipotent type of cell. Such a concept, refuted by the majority, promoted discussions and polemics that did not settle the problem (which could not possibly be settled in the absence of rigorous tests of differentiation) but helped in another respect—in making biologists more aware of the limitations and fallacies of morphological criteria, so much overrated in the past.

While the method of tissue culture was used mainly in connection with such problems as the ones mentioned above and the growth characteristics of different tissues were repeatedly tested *in vitro*, embryology was making its most impressive advances which were soon to culminate in the discovery of embryological induction. In the years that followed the memorable year 1924, the problem of induction attracted so many students that all possible avenues opened by the discovery were soon explored by enthusiastic disciples of the new creed. The obvious consequence was the rapid exhaustion of the field. At the beginning of the present decade, students of induction and related embryological problems held a rather pessimistic view of the prospects in this field. One of the leaders and most active workers in experimental embryology, Holtfreter (*2*), stated that a deadlock had been reached and emphasized the need for new ideas and new methods.

Throughout the past 20 years, workers in experimental embryology and tissue culture practically ignored each other. The embryologists have manifested little interest in tissue culture. Nor have the tissue culturists made much use of the possibilities of their techniques in dealing with embryological problems. Holtfreter in some pioneer excursions and the Strangeways school in certain studies of morphological problems by tissue-culture methods are exceptions. There are two main reasons for this lack of interest: 1) in both fields students were too much preoccupied

with their own problems to seek others, 2) the structural simplification that occurs *in vitro* in the explanted tissues discouraged the use of this method in connection with morphogenetic problems. It was only when the two methods seemed to have exhausted their own tools that excursions into the other fields became more frequent, embryologists divorcing the embryo and studying embryological problems *in vitro* and experts in tissue culture becoming acquainted with embryology.

A survey of the literature in this borderline field shows that interest is now converging on problems of embryonic induction and tissue interactions. The results will be considered in another section. In this section, I should like to present for discussion an experimental analysis of neural-crest derivatives. The first part will deal with the investigation by Twitty and Twitty and Niu on pigmentation patterns and pigment formation in Amphibia. The second is a short survey of the problem of pigment synthesis *in vitro*, and the third is based on work on nerve-cell differentiation *in vivo* and *in vitro*.

In Vivo and *In Vitro* Analysis of Pigment Patterns in Amphibia

This research, which stemmed from experimental embryology and never lost contact with the embryo did not suffer from the general trend of setting aside the embryo in favor of embryonic tissues or cell agglomerates. Perhaps in no other instance were the two methods more successfully combined in the solution of a developmental problem.

Throughout the investigation, which continued for a number of years to the present, experiments *in vivo* and *in vitro* were synchronized and interwoven in such a way as to make difficult a separate analysis of the 2. It is not, however, the scope of this paper to present in detail these results, which were recently reviewed by Twitty (*3*, *4*). I should like, rather, to consider the several steps of the investigation and the mode of attack upon the problem with the tools of the 2 techniques, so skillfully handled by the authors.

It has been stated that the success of research depends on the ingenuity of the investigator as much as on the right selection of the object of analysis. This is fully supported by the investigation that I want to discuss. The research centered on the analysis of migratory movements of immature pigment cells in *Triturus* and on the subsequent process of pigment formation in different species of Amphibia. It may be worth recalling that in these cells the processes of migration and full differentiation occur in separate and sequential phases. The spatial and temporal patterns are defined to a degree unmatched by any other cellular groups. The choice was particularly fortunate in other respects also: Different species exhibit variations in these patterns as well as in the number of chromatophores and in the amount of pigment synthesized by individual cells. Such differences afford excellent opportunities to the investigator for testing the mutual dependence of these populations and cell characteristics and the effect of environmental factors *in vivo* and *in vitro*. Finally it should be mentioned, as additional assets, that the large size of the

cells, their peculiar shape, and their remarkable resistance to manipulation makes them particularly apt to experimental analysis.

Well aware of all these advantages, Twitty, joined some years later by Niu, investigated all the aspects of the problem, leaving practically no question unanswered. At first a simple experiment was designed to explore the migratory activity of chromatophores *in vitro*. Previous observations *in vivo* had suggested that the migration of these cells from the neural crest was directed by environmental differentials in the medium.

The problem arose of the hypothetical factors involved in such motility. The method *in vitro* seemed to offer better opportunity to investigate this problem than the experimental analysis *in vivo*. Briefly, the experiment consisted of explanting fragments of neural tube and neural crest in capillary tubes filled with coelomic fluid extracted from adult newts. The tubes, sealed at one end only, were immersed in petri dishes filled with the same solution. Soon, chromatophores migrated from the explant. The first outgrowth was directed toward the open end; outgrowth toward the sealed end started sometime later, but continued considerably longer. These results are readily explainable if we accept the hypothesis that the cells move in response to mutual stimulation or repulsion mediated through the action of substances released by the cells. There is a continuous exchange at the open end between the fluid filling the capillary tube and the solution in the container. At the sealed end diffusible substances released by the cells are retained. This explanation is strongly supported by arguments which, for the sake of brevity, will not be repeated here (*5*).

Although such an interpretation could explain the migration and scattering of chromatophores in forms like *Triturus rivularis*, it seemed at first difficult to reconcile with the observation that in other species, like *T. torosus*, the same cells aggregate in a pair of compact, dorsal stripes. Experiments *in vivo* and *in vitro* solved this apparent contradiction. It was observed that in both species, the chromatophores are evenly scattered when they first migrate from the neural crest; subsequently a reaggregation occurs in *T. torosus* but not in *T. rivularis*. The reaggregation, states Twitty, ". . . is dependent upon changes; probably the retraction of interconnecting processes, which materialize only with the attainment of a requisite degree of melanophore differentiation . . ." (*6*).

The correlation between the 2 processes, cell reaggregation and degree of melanization, was manifest in explants of neural crest of *T. torosus* and to a lesser extent of *T. rivularis*. The spreading of immature cells from the explant and the subsequent reaggregation of chromatophores with the onset of melanization is so clearly documented in the microphotographs and drawings that no further elaboration is needed (fig. 1, plate 50). The difference in behavior of melanoblasts in the 2 species is explained by the inherent differences of melanin content in the cells of the 2 species. Simple inspection shows that pigment cells are much darker in *T. torosus* than in *T. rivularis*. Heteroplastic transplantations

from specimens of one to specimens of the other species proved that such differences are controlled by genetic and not environmental factors. However, a latent capacity for more intense melanization and reaggregation exists also in chromatophores of *T. rivularis;* given the right conditions, chromatophores of this species show more intense pigmentation and a certain degree of reaggregation *in vitro* (*6*). Conversely, chromatophores of *T. torosus* reared in media that do not provide the conditions for full differentiation (physiological instead of coelomic fluid) never become fully pigmented nor reaggregate *in vitro.*

In the following years, other ingenious experiments were devised to test the effect of "population pressure" and of environmental conditions on the migratory activity of the cells and the degree of pigmentation. Lack of time prevents me from referring to the numerous and most interesting experiments that again prove the value of applying a combination of the two methods to embryological problems. Briefly the results may be summarized as follows:

Chromatophores leave the neural crest and migrate in a lateral and ventral direction in a scattered fashion. Diffusible agents released by immature cells promote their migratory activity and keep the cells at a distance from each other during the migratory phase. The most convincing evidence of the role played by these substances (still unidentified) in arousing cell motility, came from experiments performed with individual or paired cells isolated in small capillary tubes. In complete isolation, chromatophores remain essentially stationary, executing at best, some oscillatory movements which do not result in effective displacement of the cell. If isolated in pairs, the cells move away from each other (fig. 2, plate 51). Their motility is further enhanced if, instead of 2, a small group of cells is explanted in the tube (*7*).

The significance of these findings extends well beyond explaining the migratory patterns of chromatophores in Amphibia. Preliminary experiments by the same authors suggest that the same mechanism is involved in the motility of mesenchymal cells. It may be interesting to mention that, long before these experiments were performed, Burrows (*8*) had elaborated a similar hypothesis of motility of embryonic cells. This hypothesis, however, sounded too fantastic to receive consideration by his contemporaries. The reasons for general acceptance of the theory proposed by Twitty and Twitty and Niu, lie in their rigorous and convincing experiments and in changes in thinking, through the years, which now favor belief in such "diffusible agents." It is worth recalling, that impressive evidence of the existence of such substances and of their role in morphogenetic processes also was demonstrated in recent years in other rather remote fields of biology. Experiments by Bonner (*9, 10*), further extended by Raper (*11*) and by Sussman (*12, 13*), proved beyond doubt that the aggregation of myxamoebae is directed by substances released by the same microorganisms. I hope Dr. Raper will tell us about this remarkable morphogenetic effect.

The second part of the investigation by Twitty and Niu on pigment

synthesis in chromatoblasts and the dependence of this process upon genetic and environmental factors is of no less importance and heuristic value. Competition of pro-pigment cells for the essential substrate was clearly documented in experiments on heterotransplantation, on homotransplantation between specimens at different developmental stages, and on the effect of drugs inhibiting melanin synthesis and differentially affecting this process in melanophores of different species. This experiment, extended by Dr. Wilde with a somewhat different approach, brings us to the consideration of another problem, which will be the object of the second part of this discussion.

Analysis of Some Differentiative Processes *in Vitro*

I shall consider here 2 experiments that illustrate the recent trend of investigating differentiative processes *in vitro*. Since the authors of these investigations are present, I will limit myself to presenting the problems and ask them to comment on the results and perspectives in the field.

Both investigations centered on the problem of differentiation of neural-crest derivatives *in vitro*. The divergence of many cell lines from common precursors in the neural crest poses the same problems as the emergence of various cellular types from the fertilized egg. The limited variety of cells originating from the neural crest, as compared with the wide range of cells from the egg cell, and the spatially and temporally defined patterns of differentiation in neural-crest derivatives, make these cells particularly favorable objects for analysis. Two complementary techniques were used: In the first, labeled substrates were added to the culture medium and their degree of incorporation was ascertained by autoradiography. In the second, the effects of amino acid analogues on differentiation of neural-crest derivatives were explored. Both techniques have been used successfully in recent years in studying microbiological and embryological problems, and there is no doubt of the potential value of such techniques.

The usefulness of a new technique can be judged from its contributions to the problem investigated as well as from its success in revealing the weaknesses of old methods and the fragility of the foundation of generally accepted principles. The investigation by Dr. Markert proves this point. The problem was to identify the initial substrates used by melanoblasts in the synthesis of melanin. The failure of tyrosine and DOPA, labeled on the side chain, to accumulate selectively in the melanin granules synthesized *in vitro* came as a surprise to the author and to the reader and cast doubt on the role of these substances as precursors. Similar negative results were obtained with labeled tryptophan, which had been claimed as a precursor of yellow pigment. Positive results were obtained with uniformly labeled tyrosine. At the time Dr. Markert reported this (*14*) research was still in progress. I hope he will enlarge on these results as well as on other aspects of his investigation of cell differentiation with isotopic tracers.

Dr. Wilde (*15*) has also explored the effect of amino acid analogues on differentiation of melanophores arising from neural crest.

The experiments to be summarized and discussed here were designed to analyze some aspects of metabolic processes in cells originating from the neural crest. The use of amino acid analogues was based on the assumption that different analogues would exert a differential effect on such cells. Analogues of phenylalanine and of tyrosine were added to the medium at concentrations varying between 1 and 0.125 m*M* per l. All the experiments were performed on pure neuroepithelium isolated from *Triturus*. The effects elicited by the several analogues were varied but well in line with the hypothesis mentioned above. Addition of atoms or groups to the phenylalanine ring resulted in a general inhibition on all neural-crest derivatives. Specific inhibitory effects on various types of cells (Schwann cells, fibroblasts) were observed when the medium was supplemented with heterocyclic 5-membered analogues of the same amino acid. In a parallel series of experiments with ventral ectoderm, the addition of phenylalanine to the culture medium resulted in 1) anomalous differentiation of pigment cells from cells that normally would not synthesize pigment, and 2) the appearance of cells that Dr. Wilde described as "strange mesenchyme."

These provocative results, while indicating a promising approach to the problem of cell differentiation, raise questions and call for some comments.

When cells migrating from the explant remain "plump and rounded," as in the instance mentioned in this investigation, can we speak of cells which "never completed their differentiation" or may it not be that the anomalous shape and appearance betray a toxic and nonspecific effect of the analogue added to the medium? In their experimental analysis, Twitty and Niu relied on the advantageous peculiarity of pigment cells. The presence or absence of melanin can, in fact, be considered a valid criterion for discriminating between an immature and mature melanophore. But this is not the case with other neural-crest derivatives, like fibroblasts, Schwann cells, and, even to some extent, nerve cells in earlier phases of differentiation. Can we, with reasonable certainty, recognize a Schwann cell from a fibroblast gliding along a nerve fiber? What is the difference between a melanophore and a xanthophore before the onset of pigmentation? Which types of cells would have normally differentiated from "strange" mesenchymal cells?

The fallacy of using only morphological criteria (and at present these are still the only ones we rely on) has been noted since the first experiments *in vitro* were performed. The heated polemic on whether cells can dedifferentiate *in vitro* and be channeled into new pathways of differentiation has been mentioned. In this respect we are in no better position now than we were 20 years ago. It may be pertinent to mention in this connection the results reported by Herrmann who also made use of—β-2-thienylalanine—the same analogue which was investigated by Dr. Wilde (*16*). He wanted to test the effects of this analogue on morphogenetic processes in chick embryos during the first 2 days of development. Embryos, 24 hours old, were removed from the egg and explanted *in toto in vitro* by the technique suggested by Spratt. The experiments were terminated 18 hours later. Distinct abnormalities were observed in

some morphogenetic processes, such as the formation of small, irregularly shaped somites and malformations in the central nervous system.

Clear-cut morphogenetic effects by antimetabolites were also described by Hörstadius and Gustafson, who investigated morphogenetic processes in sea urchins (*17*, *18*). The question was raised by the authors whether the observed effects were due to antimetabolic interference with biochemical synthesis or to some nonspecific effect of the chemicals used. Two of the antimetabolites tested—8-chloroxanthine and β-phenyllactic acid—have a surface activity similar to the detergents Duponol® (dodecyl sodium sulfate) and Texapon® (lauryl sodium sulfate) which produce similar morphogenetic effects on sea urchins. The possibility was therefore suggested that both groups of substances affect developmental processes by the same nonmetabolic mechanism. In commenting on these and similar experiments Waddington, Feldman, and Perry (*19*) write: "It is clear that embryological studies employing metabolite antagonists which one may hope will operate on definite biochemical processes, as well as investigations using labeled metabolites, are both in their infancy."

Such acknowledgment, of course, should not deter further experimentation but warns against too hasty interpretations of the observed cytogenetic and morphogenetic effects.

Differentiative Processes in Nerve Cells under Normal and Experimental Conditions

This section presents some aspects of differentiative processes taking place in sensory and sympathetic cells of chick embryos. They were the object of two parallel series of investigations performed by the writer in association with Dr. Hamburger and Dr. S. Cohen.

In the experiments mentioned in the first part of this paper, the method of tissue culture offered a precious tool for exploring some aspects of morphogenetic and differentiative processes. Those briefly summarized in the second part illustrated the use of the method *in vitro* in connection with other facets of the problem of cell differentiation. In the experiments to be reported here, the two methods of experimental embryology and tissue culture were used concurrently to compare the *in vivo* and *in vitro* effects of growth-promoting agents on nerve cells.

The simplified conditions offered by the method of explantation *in vitro* provided, as in the experiments by Twitty and Niu, the possibility of investigating some aspects of the problem not amenable to analysis in the embryo. The method *in vitro* was also useful in another respect. Nerve cells, more than any other cells, are dependent for their development and growth upon environmental conditions, since they establish contact, through their neurites, with other tissues. By explanting nerve cells *in vitro* and preventing the formation of these connections, one can learn about the influence exerted by organismal factors on nerve-cell development under normal and experimental conditions.

The experiments in question already have been the object of numerous reports and will therefore not be recounted here but only summarized to the extent necessary as a framework for discussion.

The remarkable and most unexpected effect of mouse sarcomas 180 and 37 on sensory and sympathetic ganglia of chick embryos was reported in earlier investigations (*20*, *21*). Fragments of these tumors, when transplanted to the body wall of 3-day-old-chick embryos grow vigorously and are invaded by sensory and sympathetic fibers emerging from the adjacent ganglia. The ganglia undergo considerable enlargement, which is the over-all result of an increase in cell number and cell size and of the accelerated speed of differentiative processes of individual neurons. This appeared to be comparable to the enlargement of ganglia upon transplantation of an additional limb. In both groups of experiments the peripheral field was considerably enlarged as a result of the operation. In both instances nerves emerging from the ganglia branched profusely in the grafted tissue. The increase in size of the ganglia supplying nerves to the tumor was therefore considered another evidence of those vaguely defined "peripheral effects," so often mentioned in neurology but so little understood. Further experiments, however, revealed new aspects of the phenomenon that did not fit this hypothesis: 1) Sympathetic ganglionic masses, which have no parallel in control embryos, were found adjacent to and embedded in the implanted tumor; 2) sympathetic ganglia, distant from the tumor and not participating in its innervation, were also highly hypertrophic and hyperplastic; 3) viscera normally devoid of nerves at corresponding developmental stages were literally flooded with nerves; 4) sympathetic nerves were found not only in the viscera but also inside the veins of the host, where they gave rise to large neuromas bulging into the blood stream and partly obliterating the circulation (*22*, *23*).

These perplexing phenomena could certainly not be explained as resulting from an "enlarged periphery" and suggested that we are dealing with a diffusible growth-promoting agent released by the tumor. This hypothesis was tested by investigating the effects of implanting the tumor on the allantoic membrane of the embryos. In this position the tumor could not be reached by nerves of the host. The results of this experiment were identical with those of the intra-embryonic transplantation experiments and strongly support the hypothesis previously mentioned (*22*).

The question arose as to the primary target of the hypothetical agent. Were the nerve cells the target or did the entire embryo, permeated by this "agent," become abnormally permeable to sympathetic nerve fibers? The tissue-culture method seemed to offer the best way of deciding between these two alternatives. Sensory and sympathetic ganglia were explanted *in vitro* in proximity but *not in contact* with fragments of mouse sarcomas. The standard hanging-drop method was used. The results clearly favored the hypothesis of direct action of the tumoral agent on nerve cells; ganglia, exposed *in vitro* to the effect of vigorously growing tumor produced an exuberant growth of nerve fiber tissue (*24*). The

agent released in the culture medium obviously "forced" the cells to produce more fibers than they would have produced otherwise. As in the experiments *in vivo*, it was apparent that the tumor also affected other aspects of fiber outgrowth. The nerves emerging from the ganglia were not only much more numerous than in the controls but their rate of growth was remarkably enhanced and their mode of growth strikingly different from normal, as documented in figures 3 and 4, plate 52. Subsequent experiments with a cell-free homogenate of the tumor added to the culture medium reproduced the effect elicited by the living tumor (fig. 5, plate 52). Such results proved that the "agent" could be extracted from the cells and opened the way to a biochemical approach to the problem. The isolation and identification of the agent became the immediate goal of the research. A heat labile, nondialyzable factor was isolated from the tumor. Experiments *in vitro* proved that it still retained all the growth-promoting activity of the growing tumor. Chemical analysis of the active fraction indicated that it is a protein or a protein-bound particle (*25*).

While research was in progress, another growth-stimulating agent was discovered quite unexpectedly in an unrelated biological material. It was found that the crude extract of rattlesnake venom duplicates in such a remarkable way the *in vitro* effects of the tumoral agent as to be practically indistinguishable from it (fig. 6, plate 52). Later other snake venoms were tested and found equally effective. The two effects differed in only one respect—the one elicited by the venom is much more potent. A comparison of the effects elicited by the purest available fractions of the sarcoma and of the snake venom proved that the latter is about 1,000 times as effective as the former, on a dry-weight basis (*26*).

The striking similarity of these results suggested that the purified fraction of the venom should be tested on the embryo. To this end, the active fraction was injected in minute amounts into the yolk sac of 6- to 10-day-old chick embryos. The toxicity of the venom was reflected in the high mortality of the embryos. However, a certain number of specimens survived for 3 to 4 days. In all the cases examined, the venom had elicited effects identical to the ones elicited by the growing tumor, that is, enlargement of the sympathetic and spinal ganglia and neurotization of viscera, including the penetration of nerve fibers into blood vessels (figs. 7–10, plate 52) (*27*).

The similarity in biological properties of the 2 agents was paralleled in their chemical characteristics. Analysis of the active fraction isolated from the venom indicated that we were dealing again with a heat-labile, nondialyzable factor, which was identified as a protein-bound particle.

The possibility was suggested that both factors display a common enzymatic activity on nerve cells (*27*).

The data, briefly summarized here, were presented because they are of interest in connection with the problems discussed previously. A few lines of comment may be appropriate.

By comparing the *in vivo* and *in vitro* effects of the tumor and the

venom (and in view of their striking similarity it is immaterial whether we refer to one or the other), we gained new perspectives on the processes of nerve-cell differentiation and of fiber outgrowth. In this respect, these experiments were similar to the ones described in the first part of this paper. In addition, they disclosed some perplexing features of these processes, for instance, the possibility of "speeding" some normal differentiative events and of channeling nerve fibers into abnormal routes. It was no less perplexing to learn that the agent causing such aberrations is, in both instances, a protein or a protein-bound particle.

How can we visualize the mode of action of the "particles" on differentiative processes of nerve cells and the mechanisms that are set in motion in the same cells?

If we could answer these questions, we would have solved one of the basic problems of cell differentiation and morphogenesis.

The finding not of an answer but of a promising lead toward the solution of these problems is a rewarding result, which we owe to the combined methods of experimental embryology and tissue culture.

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PLATE 50

FIGURE 1.—Successive stages in the outgrowth and reaggregation of *Triturus torosus* melanophores on the surface film of a drop of coelomic fluid. *A*, 3 days; *B*, 4 days; *C*, 6 days; *D*, 10 days; *E*, 12 days; *F*, 14 days after explantation. Curved line at *right* in *D*, *E*, and *F* represents the margin of the drop. (*From* Twitty: J. Exper. Zool., 1945, p. 155.)

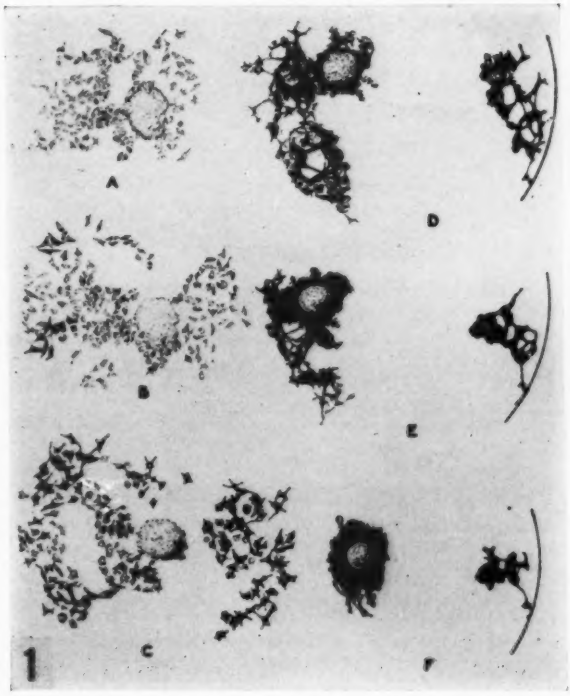

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PLATE 51

FIGURE 2.—Migratory behavior of pro-pigment cells when isolated singly or in pairs. 3 *a*, *b*, and *c*: A few hours, 2 days, and 8 days, respectively, after isolation. Debris attached to tube serves as fixed point of reference. *d*, Same cell at higher magnification 6 days after isolation. Melanin synthesis has begun. 4 *a*, *b*, and *c*: A few hours, 1 day, and 1½ days, respectively, after isolation of 2 cells. (*From* Twitty and Niu: J. Exper. Zool., 1954, p. 551.)

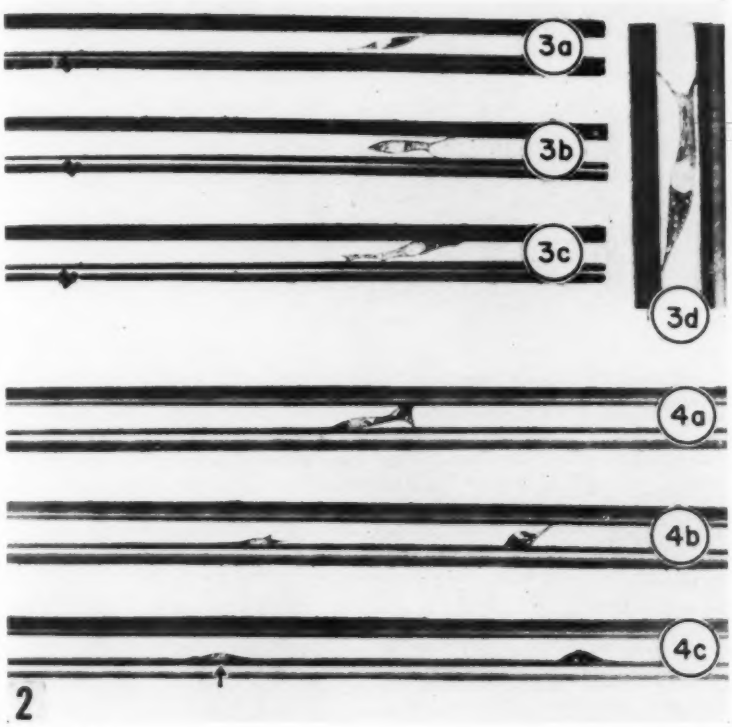

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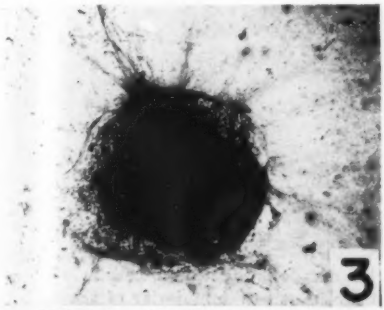
PLATE 52

Figures 3, 5, 7, 8, 9, 10 reproduced from Proc. Nat. Acad. Sc. 42: 690, 1956.

FIGURES 3 to 6.—Photomicrographs of silver-impregnated sensory ganglia comparing the effect of the intact tumor (sarcoma 180), the cell-free extract of the tumor, and the snake venom on sensory ganglia *in vitro*. All ganglia were isolated from 7-day-old embryos and cultured for 24 hours. *Figure 3*, control ganglion; *figure 4*, ganglion combined with a fragment of mouse sarcoma 180; *figure 5*, ganglion growing in a medium to which the cell-free extract of the tumor has been added; *figure 6*, ganglion growing in a medium to which the purified fraction of moccasin venom had been added.

FIGURES 7 to 10.—Photomicrographs of silver-impregnated control and experimental embryos. *Figure 7*, prevertebral sympathetic ganglion of a 10-day-old embryo; *figure 8*, prevertebral sympathetic ganglion of a 10-day-old embryo injected with the purified fraction of moccasin venom; *figure 9*, neuroma projecting into the lumen of a vein of a 9-day-old embryo with an extra-embryonic sarcoma 37; *figure 10*, same in a 10-day-old embryo, injected with the purified fraction of moccasin venom.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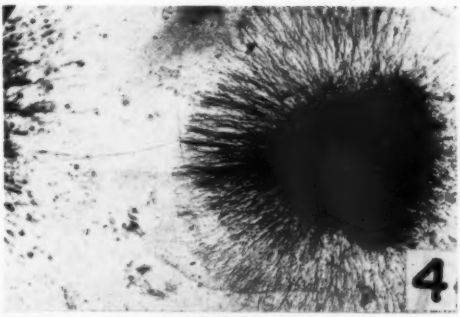
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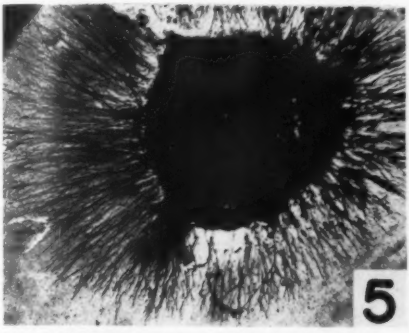
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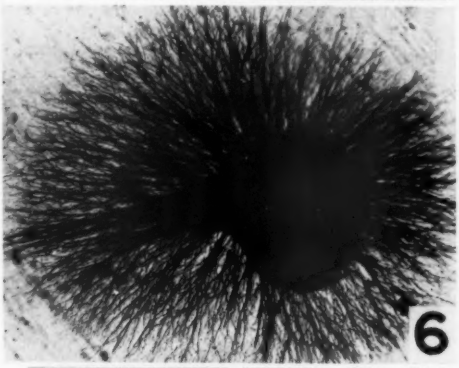
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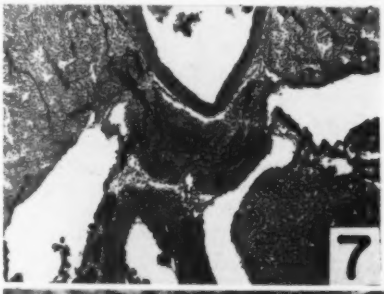
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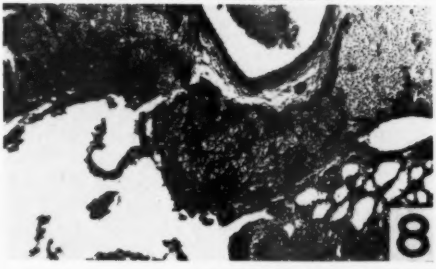
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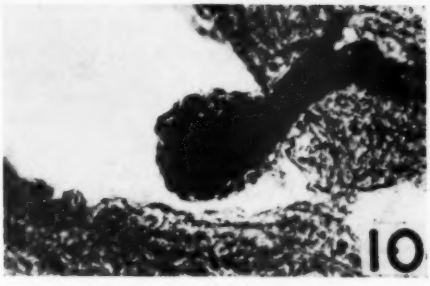
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DISCUSSION

Dr. Bloom: We all know that tissue culture has many facets and that they reflect the different interests, backgrounds, and technical capabilities of the various investigators. The methods have ranged from the simple explants of Harrison and the early work of the Lewises to the more complex techniques that developed gradually and are so widely used today, including those for the large-scale production of cells. It is probably true today, more so than a few decades ago, that to the majority of people using it, tissue culture represents one of several tools for the study of biological problems; for others, it is the main tool.

We have just heard of the effective use of the interplay of tissue culture and *in vivo* experimentation in the attack on some problems of development and differentiation, and we have seen some striking examples of differentiation in tissue culture. Sometimes tissue culture, with or without the use of complementary experiments in the body, has been used in attempts to explain some of the things that have been observed in the body. Conversely, experiments *in vivo* have occasionally helped explain what is seen in culture. I am reminded by Dr. Gaillard's presentation of two sets of experiments on bone. I refer first to the beautiful, simple work that Barnicot, of London, did some 5 or 6 years ago in elucidating, almost as well as we know it today, the mode of action of the parathyroid gland on bone. Before his work there was a strongly held view that, by increasing the renal excretion of phosphate, the parathyroid acted indirectly on bone to raise the blood calcium. Others believed that something elaborated by the gland acted directly on bone, a conclusion which is inescapable after Barnicot's experiments. He placed parathyroid glands onto bits of bone, which he then introduced into the brains of rats and mice, and found that a hole developed in the bone beneath the graft. In his experiments most of the bone was dead. A slight extension of this work was made in my laboratory by Chang who grafted parathyroid onto the living parietal bone of rats and mice. She also did many control experiments. She found, as Barnicot had, that bone immediately beneath the graft was resorbed. The latest stage in this progression is the work of Gaillard who added parathyroid to bits of bone in tissue culture and also found the same dissolution of bone beneath the graft.

The other experiment of which I was also reminded was that of Heinen who obtained no bone by continued subdivision of cultures of periosteum of young rats. There was an exuberant growth of spindle-shaped cells but no intercellular substance was apparent. When such cultures were introduced into the anterior chamber of the eye in contact with the iris, within 4 days a beautiful chondroid tissue developed from the culture and in 5 or 6 days bone began to form. Most interestingly, after some 30 or 40 days, the implanted cultures developed into bones, with dense, laminated bone on the outside and marrow on the inside.

We need all the types of experiments. At times, as in all disciplines, certain approaches have perhaps been overemphasized and others neglected. As a histologist who has spent much of his life on problems of differentiation, it seemed to me that there was too much emphasis, at least in certain important laboratories, on only problems of growth of cells *in vitro*, which obscured the stubborn fact that many cell types were decidedly different in their potential, despite their apparent morphologic similarity in culture. I never could agree with Carrel's thesis that the body was made up of only 4 or 5 types of cells. Dr. Fell will surely agree with me, and so will Dr. Murray and many others in this audience, that many of the kinds of things they have done so beautifully could never have been done if we had all subdivided our cultures every 2 days. I raise the question because I still believe, as I did 2 and even

3 decades ago, that one of the central problems in tissue culture is the elucidation of the nature of some of the spindle cells in particular cultures. I think some of the ingenuity that has been expended so successfully on making machines for growing cells in large amounts could perhaps be diverted now into working on the problem of how to tell the cells apart. This is an important general biological problem; it is also important because of the specific task that so many tissue culturists have faced, that is, how to tell whether or not a cell is malignant.

Tissue culture has helped solve some problems in differentiation and organization of tissues and development; it has also opened up some new ones.

Dr. Raper: I would like to comment briefly upon the morphogenetic behavior of some very primitive organisms, the cellular slime molds, or Acrasieae. These do not have a morphogenesis comparable in any sense to that of the embryos that we have just seen. They do show certain stages in which parallelism of cellular behavior is suggested. In these organisms the vegetative stage is separate from the morphogenetic phase. They are therefore better tools than any other organisms for studying morphogenesis. The vegetative stage consists of free-living amoeboid cells in a state of rapid multiplication and independent growth. In a favorable culture the colony occupies the entire area of the culture plate in 2 or 3 days. The cells are then approximately equally spaced throughout the whole. Then, in response to a stimulus that arises from within certain cells in the population, transmitted to other cells that in turn amplify this stimulus, the cells flow together and form the beginning of the so-called pseudoplasmodium. Having collected into such a body, but remaining separate one from the other, they proceed to develop a fruiting structure in which roughly 80 percent of the cells become spores and 20 percent become the supporting stalk that holds the mass of spores up in the air. The pattern of the cell aggregate suggested to the earliest students of this group that some chemotactic substance probably drew the myxamoebae together. Olive, as early as 1902, was convinced of this and did some experiments to try to demonstrate it. He was not successful. Later Harper concluded that fruiting was a response to negative hydrotaxis and that the cells actually came together at the driest points in his culture plates.

Perhaps the first convincing evidence that the cells were actually aggregating in response to a substance produced by some of their numbers was the work of Dr. Thom and myself some years ago, when we planted cells of different species and genera in close proximity in the same environment. For example, in a nutritive culture of *Serratia*, a red-pigmented bacterium, we planted *Dictyostelium discoideum* and *D. mucoroides*, and at their common growth frontier the aggregates consisted of streams of each type of inflowing cells. The cells of *D. mucoroides* destroyed the pigment; the ones of *D. discoideum* retained it. Thus, we could follow the 2 species clearly. In mixed species of *Dictyostelium* the 2 kinds of myxamoebae subsequently separated and fruited separately. In contrast, if cells of different genera, *e.g.*, *Polysphondylium* and *Dictyostelium*, were intermixed so that there was a completely heterogeneous population, we found that separate aggregates developed in which the streams actually overlapped. Thus, it was evident that specific substances were being produced in the cells of these centers that attracted other cells of the same species.

Soon thereafter, Runyon showed that the substance produced by *Dictyostelium* was dialyzable, that it would pass through a viscose casing, and that to form a pseudoplasmodium it would attract unaggregated cells beneath it. Bonner, in a series of exquisite experiments, showed very clearly that a cell-attracting substance was formed at the center of such an aggregate and further demonstrated that this substance stimulated the cells around the center to produce the same substance. There was thus established a continuous gradient from the center outward—always more at the center drawing in the still unaggregated cells. Bonner gave to this substance, the name "acrasin."

More recently, Shaffer, in England, has demonstrated the *in vitro* activity of acrasin. In very careful experiments, he bathed centers, then took the washings, and applied them to unaggregated cells. The unoriented cells lined up toward the point where this substance was applied. Last June, in an issue of *Science*, two papers were published, one immediately following the other, dealing with acrasin. One was a short further

report by Schaffer on his pioneering work; the other was by Sussman, who showed that there were actually 2 substances produced by *Dictyostelium discoideum*. He referred to these as acrasin A and acrasin B. He also reported the presence of an enzyme which converts acrasin B to acrasin A. According to him, acrasin A must be present in a ratio of about 2 parts to 1 part of acrasin B in order to elicit the aggregative response. Shaffer's work has shown very nicely that the so-called acrasin is not one substance but a family of substances. For example, there is undoubtedly a *Dictyostelium discoideum* acrasin, a *D. mucoroides* acrasin, etc., and one rather different than these, a *Polysphondylium* acrasin, etc. There is little doubt now that the cells are attracted to the aggregation centers by chemotactic agents or acrasins. Bonner has postulated that the further morphogenesis of these forms (*e.g.*, sorocarp formation) is also governed by acrasin. Again by beautiful experiments, he has shown that there is, in fact, a higher concentration of acrasin at the apical portion of the developing fruiting structure throughout its development.

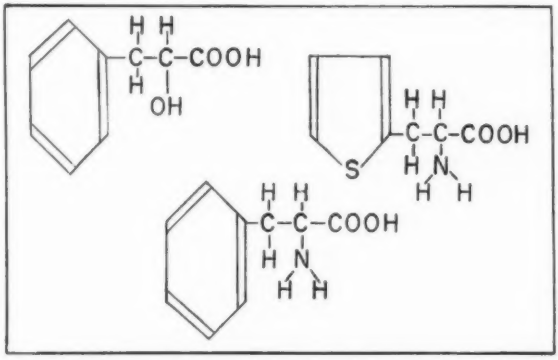
Now a word about differentiation. That too can be demonstrated beautifully in these cultures. At any stage prior to the final commitment of a cell, either to become a part of the stalk or to become a spore, the mass of myxamoebae can be disorganized, and if food is available the cells will revegetate. If there is no food, they will form secondary smaller aggregates and, in turn, produce small but typical fruiting structures. There can be no question but that many of the cells in the secondary fruiting structures then occupy positions which would have been foreign to them if they had continued to differentiate in the original structure. That is, myxamoebae initially destined to form stalk cells will become spores and *vice versa*. Thus we find in these very simple and very primitive organisms examples of a type of cellular association and organization where the function of terminal differentiation of any particular cell is not determined by the inherent characteristics of that cell *per se* but rather by its position within an integrated population of individuals that exhibit progressive functional and structural specialization as fructification proceeds. Central to this whole morphogenetic process appears to be a series of demonstrable chemical substances that arise from within the cells and subsequently control their differentiation to effect the construction of the fruiting structures, or sorocarps.

Dr. Wilde: If I may judge from this discussion, we appear to agree with regard to cellular differentiation, yet I would guess that everyone here has a particular concept which would be somewhat different from the others. For this reason, I think that it is best to refer my remarks to a particular series of cellular differentiation that I will illustrate. This precision is essential if one is to discuss experiments in which morphological criteria are used as end points and in which the known variables are chemical. The illustrations to which I refer (figs. 1, 2, 3, 4, plate 53) are photomicrographs of urodele embryonic cells. Figure 1 illustrates undifferentiated cells. They are cells that have migrated free from an explant taken at the gastrula stage. All are alike and behave similarly. They are motile, with large clear lobopodia. Cells of this type are the morphological start of our experiments. We call them undifferentiated in view of their similarity and since they will *in vitro* undergo differentiation into pigment cells and ectomesenchyme. Pigment cells are illustrated by figure 2, plate 53. These are large dendritic cells in which there has been synthesis and even distribution of melanin pigment. Ectomesenchyme (Schwann cells) is illustrated by figure 3, plate 53. This class also contains cells that differentiate as fibroblasts and melanophages. They differentiate from explanted neuroepithelium in the absence of mesoderm. To establish their origin from neuroepithelium, a series of rigid and precise surgical dissections and explantations of embryos had to be carried out and the connective-tissue cellular product traced back into the neuroepithelium. Other investigators have reported such a connective-tissue source (*1*, *2*). Since there are other well-established sources of fibroblasts, I will limit my references to those from the neuroepithelium. A product of the control cultures is seen in figure 4, plate 53. Highly differentiated striated muscle developed from cultures of cells of the archenteron-roof mesoderm, where this embryonic material was included with the neuroepithelium.

Experiments dealing with chemical causes of cellular differentiation require that a given agent be demonstrated to be effective against one cell strain (in these experiments, neuroepithelium) and not against another (archenteron roof) before specificity of activity can be imputed. Since chemical change is the probable method whereby cells do differentiate, the student of differentiation is forced to move into chemistry very carefully. Reference to the figures emphasizes that the end points of the analysis are *morphological.*

Dr. Levi-Montalcini has reviewed some of the material we have recently published (*5–6*). The central theme of this work has been the establishment, by a series of mutually supporting experiments, of a causal relationship between a special metabolism of phenylalanine and the differentiation of pigment cells and ectomesenchyme from the urodele neuroepithelium. I would like to select 1 or 2 of the experiments to illustrate both the method and the degree of specificity seen in these studies.

β-2-Thienylalanine is a specific inhibitor of the ectomesenchyme component coming from pure neural crest. It does not affect striated-muscle differentiation from the archenteron roof. Phenyllactic acid, on the other hand, is a specific inhibitor of the pigment-cell component from pure neural crest. The compound does not affect muscle differentiation. Text-figure 1 shows that the 2 compounds, although very different in structure, are analogues of phenylalanine. Speculatively, one might cut and fit the 2 molecules and thus come out with phenylalanine. Our experimental reasoning in this case was based on a similar concept. If a protoplasmic system could interchange parts, the inhibition might be relieved. If not, then with regard to the neuroepithelium the two effects of the specific inhibitors should be additive. When pure neuroepithelium was subjected to this test the latter event occurred; no cells differentiated.



TEXT-FIGURE 1.—Conventional structural formulae of phenyllactic acid, β-2-thienylalanine, and phenylalanine. Structural similarities and differences are apparent.

Surprisingly, when similar experiments were carried out on neuroepithelium and archenteron-roof mesoderm (specificity control) all cell types differentiated as expected. In other words, the system capable of relieving the 2 inhibitions did not exist in the neuroepithelium but in the archenteron roof. This experiment has been repeated and varied to test the method of action of inhibition relief by mesoderm. The ultimate chemical step appears to be the synthesis of phenylalanine (or something like it) from a phenyl-methyl-like fragment and a glycine-like fragment across an α-β bond. This agent is then transferred to the environment of the neuroepithelium, which, in turn, is able to respond by differentiation into ectomesenchyme and pigment cells. This reminds the embryologist of embryonic induction.

Analysis and study of these results have led us to conceive that the steric configuration of an active molecule—in this case, phenylalanine—is altered only with detriment

to the responding tissue. This is a crude way of approaching certain specific problems posed by Pauling (*7*).

Finally, in these as in other experiments, I do not feel that phenylalanine is the sole agent but rather that a ratio of environmentally supplied metabolites, in which a specific concentration of phenylalanine is rather crucial, is the responsible or causal factor for neuroepithelial differentiation.

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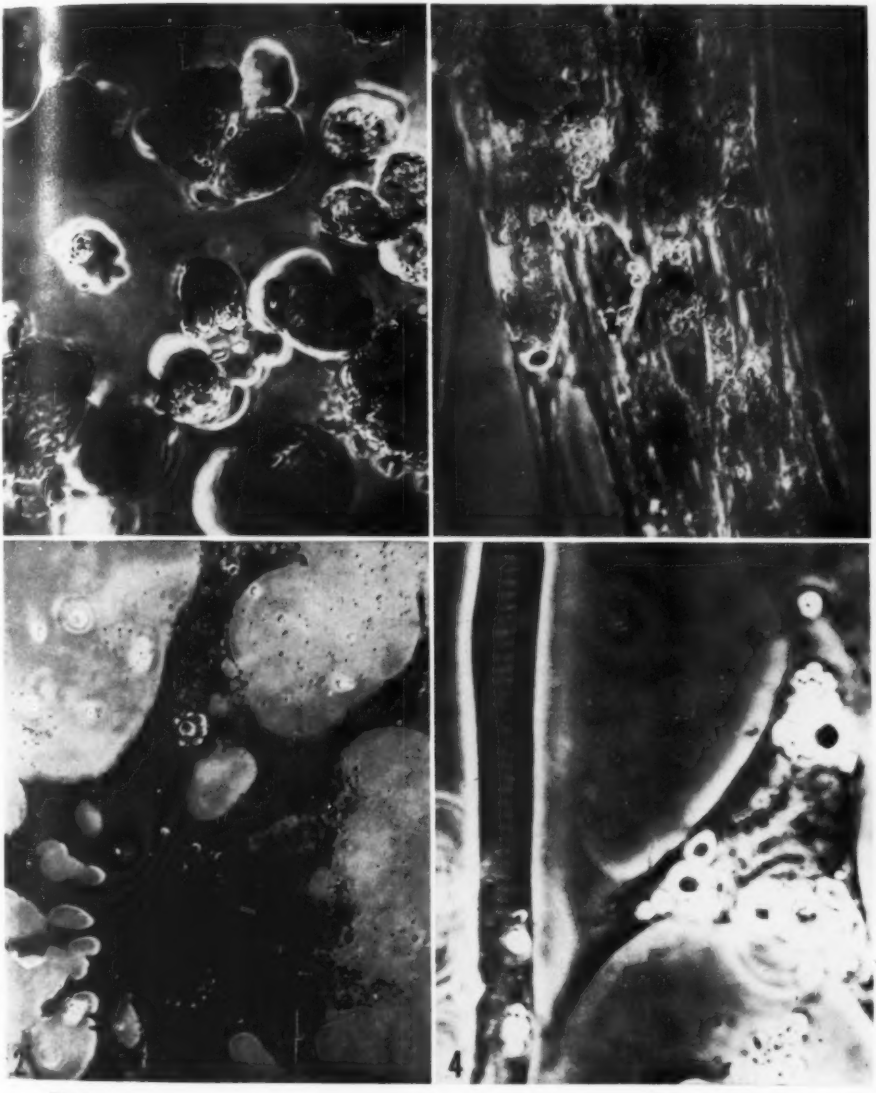
Plate 53

Figure 1.—Ameboid cells with large, clear lobopodia, all of which are similar and have migrated from an explant of urodele neuroepithelium after 48 hours *in vitro*. Explanted at stage 13 Harrison. These cells are classified as undifferentiated. × 300

Figure 2.—Mature dendritic pigment cell that differentiated from neuroepithelium explanted at stage 13 and cultivated for 10 days in nutrient medium. × 300

Figure 3.—Ectomesenchyme cells that are differentiating as Schwann cells along axones from neuroepithelium explanted at stage 13 and cultivated for 10 days. × 300

Figure 4.—Striated muscle which differentiated from explants of neuroepithelium and archenteron-roof mesoderm. Explanted at stage 13 and cultivated for 10 days. × 500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Dr. Markert: Dr. Levi-Montalcini was kind enough to ask me to extend her remarks by commenting on some of my own work, which has involved the use of tissue-culture methods in the study of certain problems of cell differentiation. Obviously in the differentiation of cells the relationship of the cell to its chemical environment is of crucial importance. There are at least two general ways in which one might examine this very important relationship. First, the environment may be altered in some known chemical way and an examination made of the consequences of this alteration on the behavior of the cells; or, second, a direct examination may be made of the normal metabolic activity of the cells in culture. We should note that Dr. Wilde's experiments, which he has just reported, are a very good illustration of the first of these approaches.

Like Dr. Wilde, I have been interested in identifying the substrate used for melanogenesis during the differentiation of a melanocyte from a melanoblast. Any one of several substances might plausibly be involved. The role of these substances may be examined by adding each substance, labeled with radioactive atoms, to cultures of developing melanoblasts. If the added substance is differentially incorporated into melanin, one may then conclude that the substance is a substrate for melanogenesis. This approach illustrates the second of the experimental procedures mentioned previously and was used with a wide array of possible substrates: tyrosine, phenylalanine, glycine, tryptophan, glucose, and a number of others. Of these, only tyrosine, labeled with carbon 14 in the ring, proved to be a substrate for melanogenesis. Seven-day-old embryonic chick skin grown in tissue culture for about 48 hours in the presence of labeled substrates developed numerous melanocytes that were not evident at the time of explantation; the cultures were then fixed, embedded in a plastic, and an autoradiographic stripping film applied to the surface of the plastic. After about 3 weeks' exposure to the *beta* rays emanating from the carbon 14, the film was developed.

Figure 1, plate 54, shows a photograph taken in the plane of the melanocytes and, adjacent to it, a photograph taken in the plane of the stripping film. Comparison of the two photographs demonstrates that the melanocytes were more radioactive than the surrounding cells. This enhanced radioactivity apparently is due to their utilization of labeled tyrosine in the synthesis of melanin.

Now, melanogenesis is only a very small aspect of cell metabolism, but by the use of labeled compounds much larger areas of cellular metabolism may be mapped out. Recently, I have been investigating some of the metabolic activities of embryonic cells in culture by identifying products made by the cells from uniformly labeled glucose added to the medium. After a period of growth the cells can be separated from the medium, extracts made from the cells, and these extracts separated chromatographically on paper according to conventional procedures. Then an autoradiogram on X-ray film may be made of the chromatogram. Compounds containing radioactive carbon atoms from the labeled sugar reveal their location on the chromatogram by inducing the formation of corresponding dark areas on the adjacent film. The identity of the radioactive compounds can be established partially by a variety of techniques, such as measurements of the relative position on the chromatogram, response to colorimetric tests, and others. These techniques were, of course, worked out by other investigators in the solution of a wide variety of problems. These techniques should now have very fruitful application to problems of tissue culture and to studies of cell differentiation.

Figure 2, plate 54, illustrates one such combination of chromatogram and autoradiogram made from chick-liver cells cultured *in vitro*. The chromatogram on the right was sprayed with ninhydrin to reveal the presence of amino acids, and it is apparent, by reference to the autoradiogram on the left, that these liver cells synthesize proline, alanine, glutamic acid, serine, and aspartic acid from uniformly labeled glucose. There are other unidentified spots on the autoradiogram that are not amino acids but that were also synthesized from the sugar. Thus we can see directly, at least in part, what these cells were doing metabolically in culture.

Similar analyses of other types of cells in culture reveal some differences among them, but there is obviously a basic core of metabolic activity common to most, perhaps all,

of the cells of any organism. Thus far, my investigations have revealed few differences but many similarities in amino acid synthesis, as shown by analyses of the contents of cells in culture.

However, comparable analyses made on the culture medium rather than upon the cells themselves reveal striking differences in the relative amounts of various kinds of radioactive products made by different types of cells in culture and released into the surrounding medium.

The autoradiograms in figure 3, plate 54, illustrate these differences, as shown by chick-heart fibroblasts and chick liver. Although both these cell types can synthesize the same amino acids they obviously release vastly different proportions of them into the medium. Such differential release of metabolites makes possible a kind of cross-feeding among the diverse cells of an organism.

When cells at different stages of embryonic differentiation are explanted *in vitro* and analyzed for their metabolic activity, it is apparent that their metabolic patterns gradually change with successive stages in differentiation. Particularly, their metabolic contribution to the surrounding medium changes. Thus, the chemical environment of differentiating cells is continually modified by the differentiating cells themselves, and these changes in the medium are probably responsible for provoking further changes in the cells. These changes in cell metabolism are of at least 3 general types: new synthetic activity becomes apparent, previous syntheses disappear, and conspicuous shifts in relative synthetic activity occur. It should be realized, of course, that the apparent synthetic activity referred to here is, in reality, a reflection of the balance between synthesis and degradation of metabolites. A change in either synthetic or degradative activity would change the quantity of a metabolite revealed by the analytical techniques used in this investigation.

Dr. Lwoff: I should like to ask a question of Dr. Wilde. What do you mean exactly by differentiation of the melanoblast into a melanophore? Is it the synthesis of the pigment or is it a permanent change?

Dr. Wilde: It is a permanent change insofar as we have studied it. You must set up stringent criteria for calling such a cell a pigment cell. We feel that such a cell should show evidence of melanin synthesis and the even distribution of melanoprotein granules throughout its cytoplasm. Furthermore, this cell should become dendritic in form and be relatively stationary. Although the criteria are arbitrary, we have felt that it is better to be very conservative in interpreting these changes. For instance, if cells become black through melanin synthesis but do not become dendritic in form, we do not consider them to be normal pigment cells. Many of these have occurred in some of our experiments and are identified by us as "anomalous pigment cells."

Dr. Lwoff: Only the melanoblast can become a melanophore? The melanoblast is already a differentiated cell, is it not?

Dr. Wilde: Yes, that is true, but we are speaking of the shift from cells for which, thus far, we have no differential morphological criteria and which now move into a particular type of differentiation. I will cite an experiment not mentioned before (Wilde C. E., Jr.: J. Morphol. 97: 313, 1955). If you treat presumptive ventral ectoderm, which will ordinarily differentiate only into skin epithelium, with a modest concentration of phenylalanine (about 2 to 8 m*M* per l.), a certain number of cells will differentiate into migratory melanoblasts. These will subsequently become pigmented and dendritic. Ventral-ectoderm cells normally form only keratinizing epidermis and have no history of becoming melanophores. However, the ventral pigment cells elicited by phenylalanine are indistinguishable from those derived from the normal source—the neural crest.

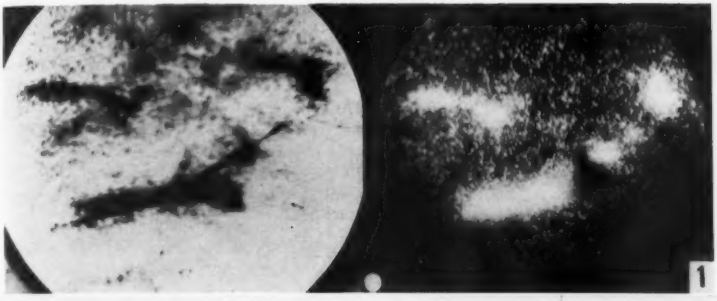
Dr. Boll: May I ask Dr. Wilde a question. I am not familiar with the details of the histology of the material you work with. You do not recognize any morphological differences between the cells with which you begin your experiments. Now suppose these cells in the original explant had been left in the tissue, in the organism. Would they have differentiated into the cells that you finally get or are you in fact

PLATE 54

FIGURE 1.—*Left:* photograph of 3 melanocytes that synthesized melanin, using labeled tyrosine as substrate. *Right:* autoradiogram of these melanocytes showing intense localization of radioactivity in the area of the melanin granules (first published in Ann. New York Acad. Sc. 60: 1010, 1955). × 500

FIGURE 2.—Chromatogram and corresponding autoradiogram of extract made from chick-liver cells after cultivation *in vitro*. *Left:* autoradiogram of the chromatogram shown on *right*. The chromatogram was developed with ninhydrin to reveal the presence of amino acids. Spots 1 and 2 are radioactive but do not represent amino acids. Only 5 of the 11 amino acids on the chromatogram are radioactive and, hence, derived from the glucose.

FIGURE 3.—Autoradiograms made from chromatograms of the media in which chick-heart fibroblasts and chick-liver cells had been grown. The glucose is unused substrate. *Note* that liver cells release into the medium relatively larger amounts of glutamate, aspartate, serine, and cysteine and relatively less proline than do the heart fibroblasts.



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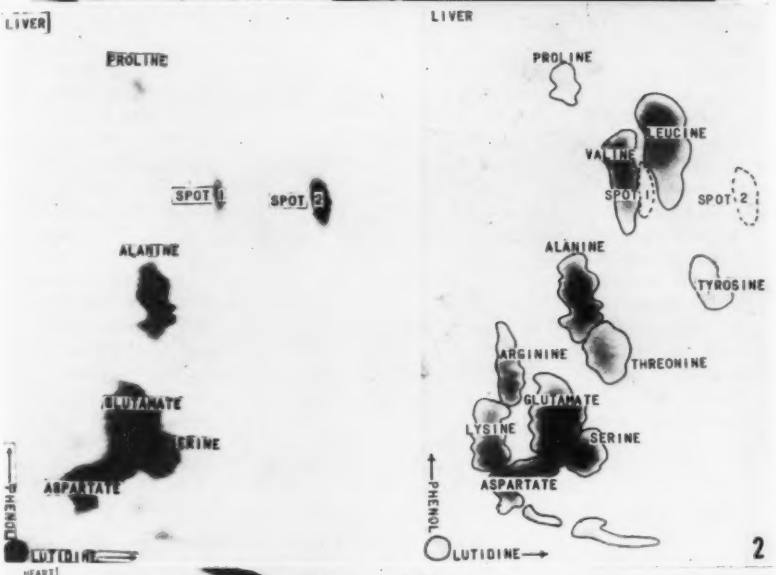

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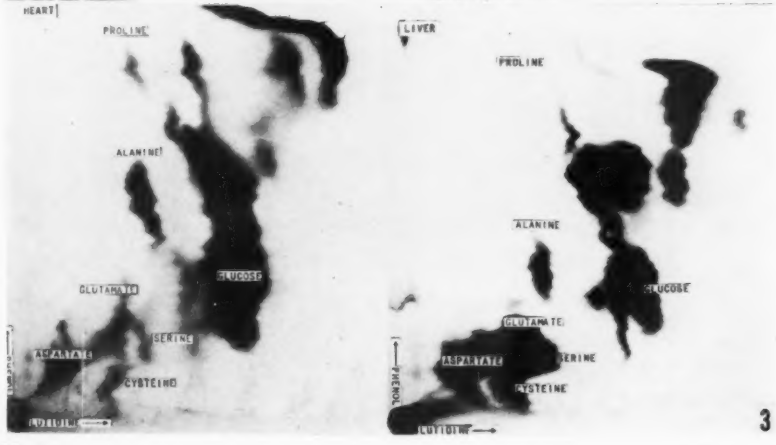

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turning the cells into something that they wouldn't ordinarily have been if they had been left where they were?

Dr. Wilde: In the experiment of which I have just spoken to Dr. Lwoff, we are, in fact, turning cells into something which they have no history, no prehistory, no apparent intention of becoming. The initial analysis with the serial amino acid analogues of phenylalanine, of course, was carried out by means of differential inhibitions and reliefs of inhibitions. One is always happy to turn the experiment around and get as a positive result an actual cellular conversion. This has been done in the experiments where the addition of a small amount of phenylalanine to cultures of ventral skin cells converted some of these into melanophores.

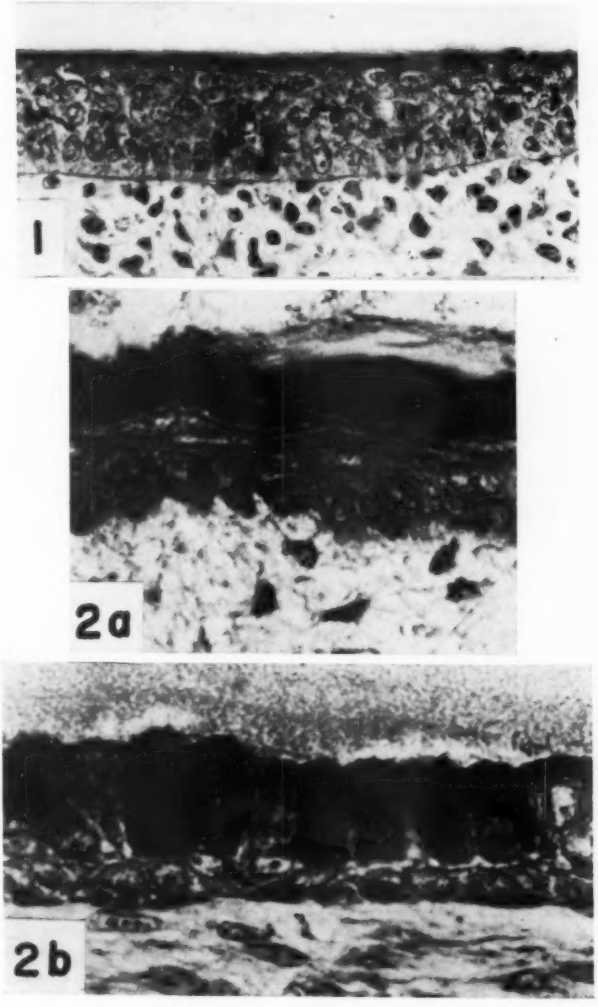
Dr. Fell: As regards the transformation of cells, the examples presented to us have represented transformations at an early stage of differentiation, into nonpresumptive tissue. Examples of transformation of tissue in very advanced stages of differentiation are given in plate 55. These experiments are really the continuation of some earlier work that the late Sir Edward Mellanby and I did on very early rudiments of chicken skin, in which we induced a mucous metaplasia by the action of excess vitamin A. In the present experiments, I have been working with the much more differentiated scaly skin from the shanks of 13- and 18-day-old chick embryos, because I wanted to see whether at this advanced stage of differentiation a metaplastic change could still be obtained. I was rather surprised to find that metaplasia could be produced perfectly well. To illustrate the transformation in the 13-day-old skin, figure 1, plate 55, shows the starting material. The scales of the leg and the quite highly differentiated epidermis that in places is just beginning to keratinize are shown. Skin of that type was grown by a modification of our standard watch-glass method, worked out by Dr. Shaffer, of England. Skin is grown on strips of cellulose-acetate cloth laid on the surface of the clot, so that it remains flat, and when the culture is fixed the cellulose-acetate fabric is dissolved out of the tissue with acetone. Figure 2, plate 55, shows the effect of excess vitamin A on the 13-day-old skin. One explant was grown in normal medium, and the squamous epithelium and the dense layers of keratin are seen; the other is from the same experiment but was grown on medium to which 1500 International Units of vitamin A per 100 ml. have been added. Typical goblet cells are pouring out secretion that stains with mucicarmine.

PLATE 55

FIGURE 1.—Cross-section of skin from the leg of a 13-day-old chick embryo, showing highly differentiated epidermis just beginning to keratinize. This is the stage at which cultures are started. × 550

FIGURE 2.—*a*) Culture, grown in normal medium, showing squamous epithelium and dense overlay of keratin. *b*) A sister culture grown in a medium containing 1500 International Units of vitamin A per 100 ml. *Note* typical goblet cells, the secretion of which stains with mucicarmine. × 550

Figures reproduced by courtesy of the Royal Society.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Dr. Grobstein: The pattern Dr. Markert has described has possible usefulness in connection with the problem of cell nutrition. Dr. Cannon some years ago used the expression "the wisdom of the body." Possibly we can speak of the wisdom of the cell and presume that the cell knows something about the kind of medium in which it likes to swim and live. With a technique such as Dr. Markert described we may get clues from the cell regarding its preferred type of medium. It seems to me that some attention might be given, by such procedures, to the question of what happens to synthetic media after cells have been grown in them for varied periods of time. One might see whether the phenomenon that vaguely has been called "conditioning" may involve addition of materials of the sort that Dr. Markert's technique may allow us to identify. I wonder if Dr. Markert would care to comment on this.

Dr. Markert: I think it would be very suitable for such studies; I have analyzed the media from cells grown in different kinds of media, ranging all the way from balanced salt solutions, in which there was, of course, no growth but some metabolism, to the most complex kinds of media involving embryo extract, plasma, etc. Although differences in metabolic activity certainly occur in these different media, I was more impressed by the similarities in metabolism than by the differences. Apparently the metabolites elaborated by cells into the medium are not in any very sensitive way a reflection of the kind of medium in which they find themselves. In compounding a synthetic medium for a given cell type it would probably be important to measure the relative amounts of normally synthesized products in order to provide a similar chemical environment which would perhaps be congenial to the cell type.

Dr. Morgan: We have published one paper on much the same ideas that have just been expressed. We prepared paper chromatograms of our used synthetic medium, following cultivation of different tissues. The chromatograms were developed specifically for amino acids. Our major work dealt with amino acid changes during the cultivation of chick embryonic-heart tissues. Here we found a characteristic pattern of uptake and accumulation of certain amino acids. We have extended these studies to other tissues, such as strain L and a variety of chick-embryonic tissues. With each cell type or tissue that we have cultivated in our synthetic medium, we find a slightly different pattern of uptake and accumulation of amino acids. In the case of the chick-heart tissue, we have correlated those changes with the essential and nonessential amino acids for that tissue. We hope to extend these studies to similar correlations as a means of developing synthetic media specific for these other tissues.

Dr. Willmer: Perhaps in such a complex situation, it may be salutary to see if there is any simple pattern or condition that might be the essential causative agent. For example, the effects of such a simple reagent as sodium chloride on ensuing differentiation may be quite surprising. I can think of two cases in particular. One concerns certain teleost fish which normally live in fresh water and which as soon as they are transferred to salt water develop on their gills, from relatively undifferentiated cells, large, round eosinophilic cells resembling oxyntic cells, and these cells apparently help in maintaining the ionic balance of the fish. The other concerns the effects of sodium chloride on certain amoebae. The particular species *Naegleria gruberi* is normally an amoeba, but if you put it into distilled water then it turns into a flagellate, which I think is a change that could legitimately be regarded as a form of differentiation. If you want to stop this change, all you have to do is to add sodium chloride. I am not suggesting that sodium chloride is relevant to the present discussion but the examples may show that simple factors of this sort should not be neglected.

Dr. Lwoff: I would like to discuss Dr. Willmer's statement that the production of a flagellum from a kinetosome is a differentiation. It would perhaps be better to call this a modulation because it is perfectly reversible. The kinetosome is there; when the concentration of sodium chloride is decreased then a flagellum is produced, but it can disappear. Is this really a differentiation?

Dr. Willmer: I agree with you entirely but I would like to know where you are going to draw the line between the two?

Dr. Lwoff: Since the flagellum is not endowed with genetic continuity the notion of differentiation or modulation is difficult to discuss.

Dr. Murray: There has been a growing impression in some quarters that the ribonucleoproteins of animal cells, or at any rate one of the ribonucleoprotein fractions, are concerned with cytodifferentiation. This concept is discussed by Brachet (p. 475) in *The Nucleic Acids*, edited by Chargaff and Davidson (1955). So far as I know, no direct chemical attack on the problem has been made; consequently, Mrs. Benitez, Dr. Chargaff, and I have been having a try at it by main strength and awkwardness. We have been encouraged by the work of Niu and Twitty, who have brought about *in vitro* the differentiation of presumptive epidermis into neural-crest tissue by substances diffusing out from organizing regions of the amphibian embryo planted in the same culture. The activity of these extracts, it appears, is largely destroyed by RNase. Dr. Levi-Montalcini's finding that growth and multiplication of neural elements in spinal and sympathetic ganglia are greatly stimulated by protein fractions from tumors, and now from snake venoms, may also have a bearing on the problem. However, she does not believe that this stimulating factor is a ribonucleoprotein. We have been encouraged as to the possibility of cell transformation by the results of Dr. Fell, who produces a mucous metaplasia in well-differentiated cornifying epidermis, albeit by the administration of high levels of vitamin A.

Our plan has been to confront cultures of adult rat fibroblasts with ribonucleoprotein preparations isolated from various organs of the adult rat. So far, we have exposed subcutaneous areolar fibroblasts to whole liver-microsomes, prepared by a method essentially similar to that of Hogeboom and Schneider (1955), and to a high degradation product of these. When whole microsomes, a lipide-nucleoprotein complex, are treated with sodium deoxycholate the lipides are removed and an insoluble fraction remains that is composed largely of dense ribonucleoprotein granules obtainable by high-speed centrifugation. This is our second experimental agent.

As a result of treatment with either whole microsomes or the deoxycholate-insoluble fraction, these adult cells are profoundly inhibited in migration and division. They are also altered morphologically, from typical fan-shaped or spindle-shaped migrating cells to sessile forms in which the nucleus is surrounded by a rather sharply circumscribed ring of cytoplasm emitting one or more long, narrow fibers of uniform width, which frequently show pseudopodial "growing-tips." This transformation is not a transient one but persists as long as the presence of the agent is continued. It becomes manifest immediately with the emergence of the outgrowth in a newly confronted culture. If either agent is applied to cells that have already emerged, they also undergo morphological transformation. (*See* figs. 1, 2, 3, 4, plate 56, of living control and treated cultures photographed in phase contrast.) This is a predictable and reproducible effect as regards both precipitating agent and morphology of the changed cells. It has occurred throughout the exposed group in 12 out of 12 experiments.

We do not yet know what is responsible for this morphological transformation. To paraphrase a sentence from the previous discussion on nutrition—we know only what we put into the medium and not what does the work. We are well aware that it may not be a specific effect of these agents. But since we have here a constant and predictable morphological transformation of adult cells, these preliminary data seemed worth reporting to this group for your comments if for no other purpose.

Dr. Waymouth: These morphological changes are very interesting. I would like to ask Dr. Murray a question about the concentration of the materials put in. Is this critical? Can you get this kind of effect with a very low concentration? One might think in terms of 1 particle per cell or something of that order. Or have you used a very heavy dose?

Dr. Murray: We use a relatively heavy dose. The effect is obtained when the microsome suspension is applied as 25 or 30 percent of the medium. As yet we can't do with less; 1 particle per cell will not do. The constitution of the carrying

medium seems unimportant; it may be simply balanced salt solution, balanced salt solution + embryo extract, or balanced salt solution + embryo extract and serum.

Dr. Leighton: Do you have data on functional changes, Dr. Murray, induced in these fibroblasts, such as the formation of collagen fibers or reticulum fibers?

Dr. Murray: Not yet. We have only been working on this for a few months.

PLATE 56

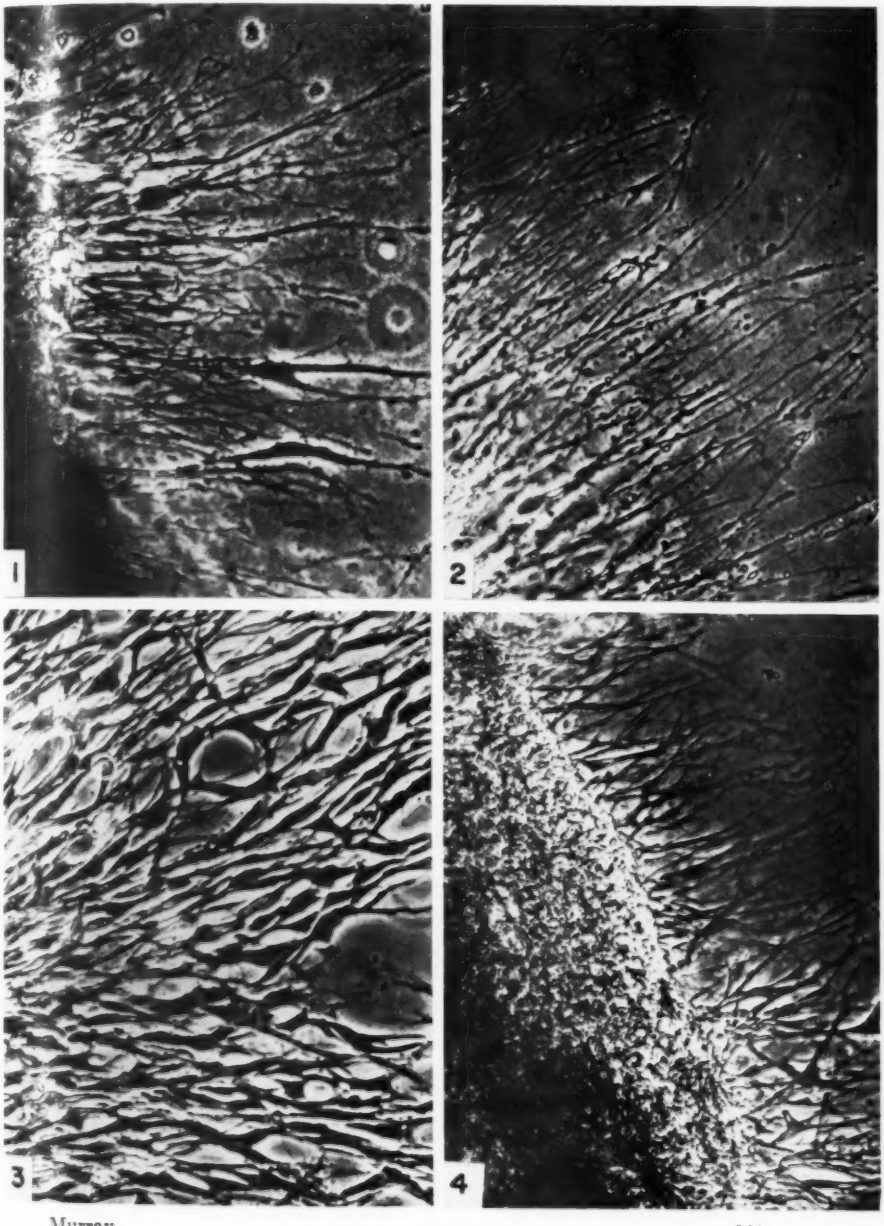
All photographs are of living cultures, phase contrast. × 250

FIGURE 1.—Adult rat fibroblasts after treatment with whole microsomes (in complete feeding solution), 6 days.

FIGURE 2.—Adult rat fibroblasts after treatment with whole microsomes (in balanced salt solution plus embryo extract), 5 days.

FIGURE 3.—Control: adult rat fibroblasts, 6 days.

FIGURE 4.—Adult rat fibroblasts after treatment with deoxycholate-insoluble fraction (in balanced salt solution alone), 48 hours.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The Future of Tissue Culture in Relation to Morphology [1,2]

HONOR B. FELL, *Strangeways Research Laboratory, Cambridge, England*

I have been asked to discuss the future of tissue culture in relation to morphology, but I think that from now on we shall see very little work done on purely morphological problems. At the present time, there is an increasing tendency in tissue-culture studies to combine morphology with physiology, and, to my mind, this is a most important and desirable trend, which I hope will become more and more pronounced as time goes on.

Although I am myself a morphologist, I freely admit that morphological research, in isolation, is a sterile pursuit. In the organism, structure and function are different manifestations of the same thing and therefore each should be studied in close relationship to the other. This is a platitude—everyone knows it to be true, and yet throughout biological research we constantly see enforced an entirely artificial separation between the two aspects of living matter.

The sterility of the purely morphological approach is well shown in the early history of tissue culture, and if we examine our excellent Bibliography, we find innumerable references to papers recording the appearance and cytological characteristics of different types of tissue grown in culture; but the results of all this labor do not add up to a very impressive advance in our knowledge of cellular biology.

Bored and disgusted with this type of descriptive morphology, biologists have been directing their attention more and more to the biochemical study of tissues. This would have been an admirable thing had not the pendulum swung much too far in the opposite direction, so that many workers were almost totally ignorant and even secretly contemptuous of the fundamental structure of the cells and tissues with which they were concerned; to them the cell was a small ampoule of assorted chemicals, which had to be cracked open so that its contents might be suitably examined in the test tube. So once more we were faced by this pernicious and unnatural divorce between morphology and physiology.

But, in our field at least, there are encouraging signs of a happy rapprochement between the two sciences. I believe that we are now entering

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an era of what we may call *physiological morphology*, and I should like to indicate what seem to me the present growing points in this subject in the tissue-culture world.

Cytology

Since morphogenetic processes ultimately depend on the structure, behavior, and metabolic processes of the individual cells of a tissue, I should like to begin at the cellular level of structure and consider what more tissue culture is likely to tell us about cells of different morphogenetic types.

The only new method for purely morphological investigation, *viz.*, electron microscopy, has so far not provided very much new information about cells *in vitro*. Unfortunately, intact cells in tissue culture are too thick for satisfactory electron micrographs of the nucleus or chromosomes, and the best pictures so far obtained show no more than can be seen in the living cell by phase contrast.

Owing to the steady improvement of the phase-contrast microscope, cytological observations on different types of living cells are likely to prove fruitful for some time to come. One of the great advantages of a tissue culture as cytological material, is that the same individual cell can be examined and photographed alive and again after it has been fixed and stained by some special cytological or cytochemical method. In this way appearances seen after fixation can be correlated with the structure of the living cell, which aids the interpretation of results and is a safeguard against drawing conclusions from artifacts produced by fixation and subsequent treatments.

An extension of such work is to combine cinematography with biophysical and biochemical methods, as has been done in Professor Randall's department by Walker and Yates (*1*). In order to obtain the synthesis curve of deoxyribonucleic acid (DNA) in fibroblastic cultures, these investigators made a cinematographic record of cell populations for periods up to 24 hours; then either they photographed the same cells with ultraviolet light and measured their nuclear absorption by a method devised by Walker and Davies (*2*) or they fixed the culture, stained it by Feulgen's method, and estimated the DNA content of the nuclei. In this way, cytochemical findings on intact cells could be interpreted in terms of the recorded life histories of the individual cells investigated. It seems to me that this kind of biographical cytology might have a good future in studies on the combined physiological and morphological effects of a wide range of experimental agents and environmental conditions.

In this connection, cultures of cell suspensions grown in perfusion chambers, which enable individual cells to be observed and photographed under various experimental conditions for days on end, are likely to play an increasingly important part. At the Strangeways Research Laboratory, we are finding the simple and efficient Buchsbaum chamber very valuable.

A new tool for the tissue-culture cytologist is the interference microscope, which not only gives a clear picture of living cells but also demon-

strates changes in their dry mass which, in the regularly shaped nucleus, can be measured in either living or fixed cells (*3*). Such measurements can be utilized in cytochemical studies; thus it is possible to "weigh" the nucleus before and after digestion with a specific enzyme, and so estimate the nuclear content of a given substance without wrecking the structure of the cell.

In the future, I hope that methods for studying both the structure and functions of cellular components in intact cells will improve in both scope and precision, and that we shall become less dependent on mechanical fractionation methods, with their dangerous sources of error, for our knowledge of the chemical and physical properties of the various constituents of the cell.

Intercellular Relationships

Turning now from the study of individual cells, I should like to consider another enthralling aspect of physiological morphology, *viz.*, the problem of intercellular relationships, for the study of which tissue culture is probably the method of choice.

Cinematography will continue to play an important part in such work. It is now recognized, however, that to make a pretty film of cellular behavior, though useful for demonstration and educational purposes, does not in itself advance knowledge very much. The work of Hughes (*4*, *8*), Pomerat (*5*), and of Abercrombie and Heaysman (*6*, *7*) has shown that in order to extract information from a film it must be carefully analyzed by examination of individual frames, usually a very lengthy and laborious process. But the results can be very rewarding and may yield valuable quantitative data about mitosis (*4*), the rate of movement of cells in relation to their mutual contacts (*6*), the way in which cells orient themselves in relation to each other (*7*), about the properties of neural tissue (*5*, *22*), and many other problems. I think there is much more to be learned from this kind of research and I hope that as many types of tissue as possible will be studied by cinematographic analysis, as this seems likely to contribute many new facts about the mechanics of tissue construction.

I am sure that the results obtained by Moscona and Moscona (*9*) and by Weiss and James (*10*) on the reorganization of differentiated tissues from cell suspensions will develop in many directions. The fact that when an embryonic tissue is reduced to a suspension of single cells, these cells are able to reaggregate to form a tissue similar in structure to the original organ, raises some interesting questions, and I am extremely annoyed with Dr. Moscona because in his beautiful paper, he has already made some of my comments on this subject old-fashioned! However, he insists that I should present my material as planned, since there are still a good many questions either unanswered or only partially answered. For example, exactly how is the histological architecture re-established? In a suspension containing both epithelial and stromal elements, do the epithelial cells cohere only with each other during reaggregation? Or do the cells cohere at random and then multiply so that adjacent nests of undifferentiated epithelial and connective-tissue cells are formed, which

then interact with one another to produce the histological pattern? Perhaps it might be possible to settle this point for certain types of tissue by the use of radioactive substances and autoradiography. It has been shown (*11*, *12*) that if some organ rudiments are subjected to a careful digestion with trypsin, the epithelial constituent can be separated from the presumptive connective tissue. The nuclei of a chick embryo could be labeled by injecting radioactive adenine or P^{32} into the egg. We could then separate the 2 components of, say, the skin or metanephros from both a labeled and an unlabeled chick at the same developmental stage, add the labeled epithelium to the unlabeled connective tissue and *vice versa*, and then disintegrate the tissues into 2 well-mixed cell suspensions. When the cells had reaggregated, the aggregates would be fixed at suitable intervals and the distribution of the labeled cells investigated by superimposing autoradiographs on the stained sections. From such preparations it should be possible to tell whether cells of the same type cohere or whether the basic cellular pattern is established at a later stage by the interaction of random groups of cells.

I think that thin sections of redifferentiating aggregates studied by electron microscopy might yield interesting results and tell us something about the first stages of the cohesion of the cells, *e.g.*, how they stick together, the early formation of intercellular materials, and the development of cellular polarity as differentiation begins.

It is certain that Dr. Grobstein's brilliant work (*13*) on induction in tissue cultures has a big future, and I am sure that this phenomenon will be investigated in many more types of organs. For instance, I wonder whether feathers, which develop readily *in vitro*, might not be interesting in this connection. We might separate the epidermis from the dermis in skin from a feather tract and from an unfeathered region, and see what happens when dermis from an unfeathered area is combined with epidermis from a feather tract and *vice versa*. Tooth and hair germs, both of which develop well in culture, might lend themselves to a similar kind of developmental analysis.

The fundamental problem in these induction phenomena, however, is the nature of the inducing substance. Dr. Grobstein (*12*) has shown that the mesenchymal rudiment of the embryonic salivary gland can induce the formation of tubules in the epithelial component of the organ, even when the two parts are separated by a porous membrane; transfilter induction of tubules in metanephrogenic mesenchyme was also produced by the spinal cord (*14*). The vital question is, what is the nature of the material that passes through the membrane? I am confident that we shall have at least part of the answer before long, because Dr. Grobstein has narrowed down the problem of induction to a point where it can at last be tackled by microchemical methods.

Effect of Metabolites on Differentiation

Closely related to this question of intercellular relationships in morphogenesis is that of the influence of special metabolites on differentiation—

another problem which is particularly suitable for investigation by means of tissue culture. The recent experiments of Wilde (*15*) are a good example of the sort of results that might be obtained. Wilde showed that the differentiation of the urodele neural crest involves the metabolism of phenylalanine. The addition of this metabolite to the culture medium caused explants of the ventral ectoderm to differentiate into pigment cells and other elements, which normally are produced only by the neural crest. Similarly, the late Sir Edward Mellanby and I (*16*) found that excess vitamin A in the culture medium causes the epidermis of 7-day chick embryos to develop into a mucin-secreting, often ciliated epithelium; recently I have found that mucous metaplasia can be produced by vitamin A even in the well-developed skin from 13- and 18-day embryos.

In some tissues, at least, determination for a given type of differentiation is really the establishment of a given threshold of response to a certain metabolite. Thus the mucous membrane of the nose maintains its secretory character at the level of vitamin A normally present in the body, but if the concentration of the vitamin falls below that threshold, the nasal epithelium becomes squamous and keratinizes. The embryonic-chick epidermis has a much higher threshold of response to vitamin A and only becomes a secretory epithelium at a level far above the concentration of vitamin that the intercellular fluid normally contains. The same principle appears to have operated in Wilde's experiments on the effect of phenylalanine on urodele ectoderm, and it may be a far more important factor in embryogenesis than we realize at present. I think this possibility should be explored and that other metabolites known to be utilized in large quantities by a particular organ should be given in excess to explants of a different but ontogenetically related organ, to see whether the tissue thus treated would be deflected from its normal path of differentiation.

When metaplasia has been experimentally produced in an organ culture, the next step is to investigate the metabolic changes associated with the morphological effects. At the Strangeways Laboratory, my colleagues and I are collaborating with Dr. Stephen Pelc in tackling certain aspects of this problem by autoradiography. The experimental and control cultures are treated with various labeled compounds, and the distribution of the radioactive material is studied by a stripping film technique (*17*). We are finding this approach very useful; for example, autoradiography has shown that the mucous metaplasia induced in embryonic epidermis by excess vitamin A involves a profound change in the sulfate metabolism of the epithelium (*18*). I think that a number of other histochemical methods would also be applicable.

Wilde's experiments (*15*) suggest that the effect of antimetabolites on differentiation might prove as interesting as that of certain normal metabolites. Wilde found that when he grew the urodele neural crest in medium containing certain analogues of phenylalanine, the formation of ectomesenchyme was specifically inhibited, although ganglion and pigment cells differentiated readily. It might well be that similar

experiments with other antimetabolites might produce developmental changes in various types of tissue and considerably enhance our knowledge of the physiological basis of their morphogenesis.

Differentiation in Chemically Defined Media

Another way of investigating the physical basis of differentiation is to study the development of various tissues in chemically defined media. This type of research was begun by Professor Wolff, who has obtained some most interesting results with fairly simple nutrients (*19*). My colleagues and I are also becoming increasingly active in this matter and for some time have been collaborating with Dr. Parker and Dr. Healy in growing organ cultures in more complex media, which permit a longer survival of the tissues than do simple solutions.

It seems probable that a chemically defined medium may have to be especially "tailored" for individual organs. For example, in Parker and Healy's medium #770 very little intercellular material develops in connective tissue, and embryonic epidermis, though it grows and survives quite well, fails to keratinize. On the other hand, in the same medium the hind-gut develops very well, forming actively contractile smooth muscle and a profusely secreting mucous membrane.

Effect of Hormones on Organ Cultures

Another very important field that is sure to receive much attention in the future is the effect of hormones on morphogenesis *in vitro*. The pioneer work of Gaillard (*20*) has already been followed by a number of studies which have shown that a wide variety of organs respond to hormonal influences when growing in culture.

In planning such experiments and in interpreting the results, several factors must be borne in mind. In the first place, we have to consider not only the effect of the agent on the explant but also the effect of the explant and the culture medium on the hormone. A negative result in experiments of this type is not very conclusive; it *may* imply that the hormone does not have a direct action on the tissue, but on the other hand, it may merely mean that the hormone is being inactivated by the culture medium. For example, Chen (*21*) has shown that insulin is destroyed fairly rapidly by embryo extract and that a much greater effect on explanted bone rudiments is obtained if extract is omitted from the medium.

When we obtain a positive result with a hormone it is not certain that this is due to the hormone in its original form, as it may have been altered, though not inactivated, by the explant or the medium. We are justified in concluding, however, that either the hormone or its derivatives have a direct action on the tissue.

As I have said, a considerable variety of morphological effects have now been produced in organ cultures by different hormones, and it should be possible to correlate some of these structural changes with the correspond-

ing alterations in the physiology of the tissue. Two of my young colleagues, Dr. Lucy and Miss Lawson, are investigating some of the metabolic effects of triiodothyronine on explanted bone rudiments which are severely affected morphologically by this agent; the results seem quite promising and I think that such work might be widely extended.

Conclusion

I have tried to indicate some of the ways in which I think that the new physiological morphology will develop, with the aid of tissue culture, during the next few years. The lines of research that seem to me particularly promising are: 1) to correlate cytological and cytochemical studies on individual cells with observations on the same cells during life; 2) to extend the experimental analysis of intercellular relationships; 3) to investigate the effects of special metabolites and antimetabolites on the morphogenesis of different tissues; 4) to study the metabolic characteristics of tissues undergoing differentiation in chemically defined media; 5) to combine morphological and physiological studies on the effects of hormones on organ cultures.

No doubt there are many other important possibilities which have not occurred to me and which will be brought up in the discussion.

I should like to end my remarks with an impassioned plea that young biologists who intend to use the tissue-culture method will first equip themselves with a sound histological training as a basis for physiological and biochemical studies. It is as stupid for a biologist to disregard the detailed morphology of the cells and tissues he wishes to investigate as it would be for a chemist to ignore the molecular structure of chemical compounds.

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DISCUSSION

Dr. Martinovitch: I wholeheartedly agree with Dr. Fell on the advantages that teamwork between morphologists and biochemists can offer for the solution of problems in experimental morphogenesis. After her admirable report on the possibilities the tissue-culture method offers for the solution of problems of differentiation of tissues and organs, I should like to present some additional experiments which the tissue-culture or, more exactly, organ-culture method may offer for the solution of problems of differentiation, growth, and the functional capacity of whole endocrine glands or fragments taken from infantile rats and cultured *in vitro*. One of these is prolonged cultivation of well-organized and relatively well-differentiated fragments of an endocrine gland such as the thyroid gland of the infantile rat. The other is the combination of the tissue-culture technique with subsequent grafting into hosts deprived of the same glands, as a check on the changes that an explanted gland may undergo *in vitro*. Such an experiment may furnish information regarding the capacity of a fragment of an organ to produce its specific hormone or hormones after long cultivation. In an experiment with the infantile rat thyroid gland, cultivated for as long as 2 months, Miss Pavlovitch, in our laboratory, added radioactive iodine to the culture medium. With the help of a Geiger counter, she found that the gland was able to concentrate considerable amounts of this isotope. By use of autoradiography she found the iodine to be concentrated in follicular cells of the gland and in the antrum of the follicle. With paper chromatography, she found that the gland *in vitro* was able to manufacture precursors of thyroxine: monoiodotyrosine, diiodotyrosine, one of the thyronines, and in some rare cases, thyroxine itself. Unlike the results obtained by Miss Pavlovitch, the work of Gonzalles on the embryonic-chick thyroid gland indicates that a cultivated fragment of thyroid loses its capacity to concentrate radioactive iodine at the 11th or 12th day (*1*, *2*).

The rat's adrenal and pituitary glands offer other examples. The adrenal gland of a 5-day-old rat will regenerate as well after the first 6 weeks of cultivation as a normal transplanted gland of the same age. Following transplantation, there is a burst of mitotic divisions in the cortical cells, the final result of which is the regeneration of the adrenal gland, which in some cases approximates in size the normal gland of corresponding age. Such explant-transplants will save the life of adrenalectomized hosts. If, however, after about 3 months of cultivation, an explanted adrenal gland is transplanted into an adrenalectomized host the transplant will take, but no dividing cells have ever been observed. It appears that after prolonged cultivation *in vitro*, the cortical cells undergo a rapid process of aging (*3*).

Some years ago we showed that hypophysectomized infantile rats would resume growth after receiving grafts of infantile pituitaries that had been cultivated *in vitro*. The animals continued to gain weight for many months (*4*).

The Severinghaus technique does not reveal the presence of *beta* cells in the pituitaries of the infantile rat. In the pituitary explants, after prolonged cultivation, *beta* cells take a distinct blue color. After 2 months of cultivation, all the granules disappear in the *beta* cells as shown by the use of the Bodian impregnation technique. Following grafting of the cultivated pituitaries, the Severinghaus technique again fails to stain these cells, but the Bodian technique clearly demonstrates that the granules have appeared again. These cells, however, differ from the normal cells in their shape and also in the number and distribution of granules. This work was done in collaboration with Miss D. Radivojevitch and Miss G. Sladitch and has not been published.

There are still other problems that could be approached by prolonged cultivation and by the combined explantation and transplantation methods. One could cite the

explantation and subsequent transplantation of whole primordia of the anterior and posterior pituitary glands with and without fragments of hypothalamus and the study of the development of the rat embryonic or infantile adrenal medulla in the absence of the cortex.

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Dr. Chèvremont: Several panel members have said that they agreed 99 percent with Dr. Fell. I agree with her not only 100 percent but even more. I, too, would emphasize that we have to combine not only studies on morphology and physiology but also studies on chemical or histochemical composition of the cells, and would like to add a few remarks based on part of the work that has been done or is now in progress in my laboratory. We must consider the influence not only of hormones or vitamins but also of enzymes. For example, the question of alkaline phosphatase was discussed a few years ago, sometimes with suspicion, but now almost everybody agrees that alkaline-phosphatase activity of the nucleus is related to growth and cell division. Biochemists have shown that beryllium almost completely inhibits alkaline phosphatase. My coworker, H. Firket, and I wondered whether beryllium could also act as an antimitotic substance. We added beryllium sulfate to cultures and observed very heavy inhibition of growth and modification of the mitosis. We have taken photographs in series and cinematographic records from living cells in phase contrast. Under the influence of Be, cells enter into prophase; the prometaphase is normal, but the mitosis is blocked in metaphase and the cell remains for 2, 4, or sometimes 6 hours in this condition—often showing heavy bubbling, which is quite visible in the cinematographic record. And suddenly the chromosomes are no longer seen but a clear area appears with 2 or 3 dots becoming darker and darker. There is a reconstruction of the nucleus without any division of the cytoplasm. Several hours later these cells look quite healthy. This takes place most frequently (58% of the mitoses) but sometimes the cell becomes binucleated or finally succeeds in dividing. Apparently, the spindle is not damaged. If you carry out the Gomori reaction in a tissue culture treated with beryllium in which growth is inhibited and in which mitosis is disturbed, the reaction is practically negative. The biochemists have also shown that magnesium has a competitive action against beryllium. If you add, at the same time, beryllium and an excess of magnesium, alkaline-phosphatase reaction is almost normal, growth is almost normal, and there is no disturbance of the mitosis. I think we may conclude that this enzyme is acting in the normal mitosis and probably it takes part in the fixation and transfer of phosphates, possibly of the phosphates of the nuclear protein. Here we have an example showing an enzyme normally acting inside the cell, but it is also possible to study experimental modifications by adding foreign enzymes to cells. I mentioned in a previous discussion that it is possible to add ribonuclease (RNase) to tissue culture and see a modification of the nucleolus of the interkinetic cell. In living cells under phase-contrast you see modifications of most of the nuclei and, taking the aspect of prophases or of beginning prophases ("pseudo-prophases"). In cultures that have been fixed and stained with toluidine blue, the remaining nucleolus or the parts of the modified nucleoli have lost their basophilia. We also made quantitative estimations of deoxyribonucleic acid (DNA) on Feulgen-stained cells with the cytophotometric methods of Pollister,

Leuchtenberger, and others. In the interkinetic nuclei, the frequency of tetraploid values is markedly higher than that in the controls. The cells blocked in prophase also have a tetraploid content of DNA. This means that the synthesis of DNA is not stopped by RNase; nevertheless, it takes place slower. Thus RNA is not *directly* involved in the synthesis of DNA, contrary to the opinion of many.

It is well known that synthesis of DNA takes place before the morphological beginning of the prophase, but we are now able to refine the term and add an appreciation of time. From other experiments (cultures cooled at 16 to 20° C. and rewarmed at 37° C.), we can draw the conclusion that, in these cultures, the synthesis of DNA takes place in about 1½ hours and is finished about 1 hour before the morphological beginning of the prophase. (Chèvremont, S., Firket, H., and Chèvremont, M.). In other observations on normal cultures, with no change of temperature (Firket), this synthesis seems to last 2 or 3 hours and to be accomplished at least 4 or 6 hours before morphological prophase.

Dr. Jacoby: Three ways of looking into the future, seem possible: by premonition, by prediction, and by what I might call "pre-meditation," with a slight alteration in the meaning of this word. The scientific validity of premonition is still *sub judice* and anyhow belongs to the realm of psychology. Predictions have already been made by the previous speakers, so I will indulge in some "pre-meditation." Furthermore, one should look into the future as it were with a squint, that is, with one eye on the past, including the present. This I should like to do, first of all, with reference to micro-cinematography. This method has come into the foreground and is likely to stay there for a long time to come. But I should like very much to endorse Dr. Fell's remark that what I might call the "slow business" of analysis must receive precedence over the "show business," as has already been done in the excellent work of Dr. Fell and Dr. Hughes on mitosis, by Dr. Gey on pinocytosis, and by Dr. Chèvremont and Dr. Frederic on the behavior of mitochondria, to mention only a few outstanding contributions. One other point comes to mind. Fascinating as movies are, their usual speeding-up is excessive and makes the gap that lies between motion and static pictures appear wider than it is or it might be. To give an example, we shall have to reconcile the extraordinary plasticity of mitochondria, as seen in motion pictures, with their almost rigid compartmental appearance as revealed by the electron microscope.

In connection with the analytical approach to the life history of cells, I regret that Dr. Willmer's serial photographic method has not been used more widely. This method is much cheaper than film and is exclusively designed for research and analysis. Though less suited for cytological studies, it is eminently suited for studies of cell populations. The rate of mitosis, movement of cells, duration of mitoses, and, above all, lengths of intermitotic periods, on which data are still very scanty, can be determined in this manner on a large number of cells.

Dr. Fell referred rightly to the "pernicious and unnatural" divorce between morphology and physiology. A similar divorce, also alarming, has occurred between histology and tissue culture, that is, between the arrangement of tissues or cells in the organism and their so-called unorganized growths *in vitro*. This gap was already evident in the early tissue-culture history when the plasma-embryo extract medium was the one most widely used; and the 3 fundamental types of tissue or cell-population pattern are well known to everyone here. This gap has, with the advent and elaboration of the fluid medium and the use of cells clinging to the glass surface, become wider still and the terms "epithelial," "fibroblastic," etc., have lost or seem to lose their meaning altogether. In fact, Dr. White's term "cell culture" is becoming more and more appropriate. But is this a desirable development? Should we not pause, at least, and probe into this changed situation? I regard it as one of the future tasks to try to bridge this gap not only by biochemical tests on these various cell strains but also by an investigation into their morphological properties, their community behavior. To what extent is this further change, "dedifferentiation" if you will, permanent, or is it still reversible, given appropriate conditions? For instance, can the "network" epithelium be returned to membrane formation? Or, to go back a step further, can it even be made to organize itself into tubules or cords or clusters depending on its origin,

if and when it is brought under the influence of suitably chosen inductors? We have recently learned from Dr. Grobstein's work that mesenchyme, after several passages in culture, will lose its inducing power by degrees. Is the responsiveness of cultured epithelium also lost? Do the so-called fibroblastic cells cultured on glass, which we know produce some sort of ground substance, produce collagen as well? Particularly, are they capable of doing so in chemically defined media? Here, the use of isotopes is indicated and may be very promising.

It is perhaps unfortunate that many of the now available cell strains are of the malignant kind. I feel that future efforts should be directed toward the cultivation of strains of normal cells, if possible, by cloning. This approach would be most valuable for answering some questions that have remained in abeyance because of the continuing argument concerning mixed-cell populations, which usually compose the original source of material: not only such questions as the possible transformation of one cell type into another but also questions of the variability with regard to various properties within a given cell type.

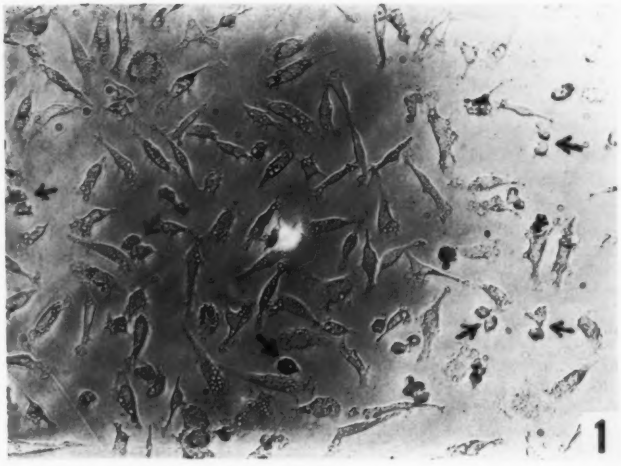
In regard to cell types, I should like to conclude with a reference to so-called macrophages. Figure 1, plate 57, shows part of a pure population of fowl macrophages with many cells in mitosis. They multiply actively in this way when cultured in dilute serum; in fact, under favorable conditions, every single cell in a given field can be seen to divide. The cells phagocytose their own kind when one of their number degenerates and dies; they take up trypan blue from solution and carbon particles from suspensions. They can be maintained in a state of active proliferation for some months, but gradually their growth rate declines, the culture becomes more and more decimated and is finally lost. The question is: What is the limiting factor (or factors) that prevents this cell type from being maintained indefinitely *in vitro*?

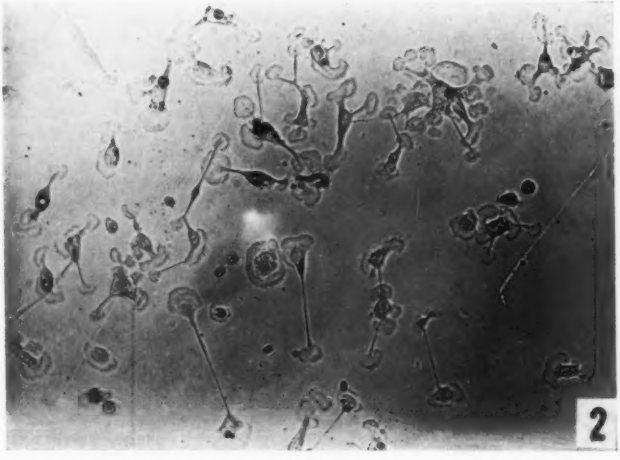
Figure 2, plate 57, shows a population of mammalian amoeboid "wandering" cells obtained from a peritoneal exudate of a guinea pig and also cultured in dilute serum, wherein they can be maintained for many weeks. In the literature they are usually

PLATE 57

FIGURE 1.—Part of a pure population of macrophages isolated from chick-embryo heart explant and now living in a Carrel flask in dilute fowl serum. Approximately 4 weeks *in vitro*. *Arrows* indicate mitoses. × 165

FIGURE 2.—Part of a population of amoeboid "wandering" cells (not lymphocytes or polymorphs) obtained from peritoneal exudate of a guinea pig. Macrophages(?) or clasmatocytes living in a Carrel flask in a dilute guinea pig-horse serum mixture. Approximately 5 weeks *in vitro*. *Note also* the many "bubble" processes characteristic of these cells, but rarely seen in fowl macrophages. The small, roundish isolated bits of protoplasm, *left lower half* of the picture, are non-nucleated bits of cytoplasm which arose through "clasmatosis," a process, again common to these cells, but not to fowl macrophages. × 180





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referred to as macrophages. But is is doubtful whether this is entirely justified. They certainly differ from the fowl macrophages in a number of respects. They do not divide readily. In a population which after fresh feeding was followed photographically for 3 days, not a single mitosis occurred. Though many cells picked up carbon particles from a suspension, they did not store trypan blue when this was added in solution to the serum as readily and speedily as do fowl macrophages.

Thus, there are here obviously two types of cell, perhaps related but certainly differing in some fundamental aspects. One is capable of proliferation, the other, it seems, represents an end- or post-mitotic form. To study further and to define more precisely the nature of these cell types, their affinities and differences, is another task for the future. All this adds up to the fact that we still have much to learn about different cell types and also about their interactions, and, for this, the tissue-culture method with all its modifications will remain one of the most valuable tools.

Dr. Steeves: Dr. Fell's stimulating remarks on the future of tissue culture in relation to morphogenesis, for me at least, have served to emphasize the validity of a conference such as this where botanists and zoologists get together to discuss their common problems. For, if our problems can be shared, it seems that our outlook is in the same direction. One might almost rephrase some of Dr. Fell's opening remarks and apply them to plant tissue culture with equal validity. The techniques of tissue and organ culture have already made possible some important advances in the realm of morphogenesis in plants; and I feel that there is no area in which the future of plant tissue culture is more promising than that which pertains to morphogenesis. Several of Dr. Fell's "growing points," have suggested certain probable directions in which plant tissue culture may also be expected to develop. I do not contend that these are strictly comparable; but at least they have been suggested by the points that Dr. Fell has made.

In the field of cytology, tissue culture provides for plant cells, as it does for animal cells, an opportunity to study cytological phenomena in cells that can be grown under carefully controlled or defined conditions. The properties of plant tissue cultures are, of course, not the same as those of animal tissue cultures, and observations on living plant cells are not so easily carried out. But the fact that cells can be grown under controlled conditions for study is a matter of great importance. I should like to mention, as an example of work of this sort, the studies of Dr. Carl Partanen in the Harvard laboratories, in which he has carried out an investigation of nuclear changes in a tumor isolated from a species of fern. This type of work was of such a nature that it could not have been done without the aid of a sterile culture technique; and I am sure that there are many similar examples. The attainment of the long-sought ideal of single cell cultures seems to render the application of culture techniques to cytological studies even more promising than it could otherwise have been.

As to intercellular relationships, I should like to suggest that in the somewhat comparable study of interactions between cells, tissues, and organs in plants, we may also see evidence of a very profitable application of the tissue-culture method. Some of the grafting experiments, which have been referred to by members of the panel in the discussion of the first section, I believe, are indicative of this. The grafting of shoot apices into root tissue or unorganized callus tissue, carried out respectively by Camus and Wetmore, have shown that the influence of these organs grafted into a tissue of another type may be studied. I think that there may well be a great deal of future work along these lines. I should also like to suggest that possibly such fundamental botanical problems as that of compatibility may appropriately be studied under conditions such as these, and there might even be some economic significance in such a study in relation to horticultural problems.

For examples of the investigation of the influences of specific substances on morphogenetic processes, I think I need only refer to the work that Dr. Skoog reported, the influence of specific substances on bud and root initiation, and also to the further work of Professor Wetmore on the effects of growth hormones and nutrients on the differentiation of vascular tissues in unorganized callus. I am certain that there will be a great future development in plant tissue culture along these lines. It might also be

mentioned that plant tissue cultures are proving to be valuable tools for the study of the effects of specific substances on such processes as cell division and cell enlargement, which do not themselves constitute what we think of as morphogenesis, but which are certainly important parts of the morphogenetic process.

In the area of organ cultures, I shall not make a point exactly comparable to that of Dr. Fell and limit this to the study of the influence of specific hormones on organs. I shall rather point to the whole field of organ culture in plants in which the cultures of organs of various types are providing useful material for experimental morphogenetic studies. Root cultures have long been studied, particularly from the physiological point of view; but we are now seeing, as Dr. Boll pointed out this morning, a turning toward the study of morphogenetic problems such as those carried out by Dr. John Torrey at Berkeley. In the culture of the shoot apex, there are still many problems to be solved, and we cannot culture apices of all plants as we would like to; yet the future holds great promise for the application of this type of culture technique to the solution of morphogenetic problems. Professor Wetmore has already begun to apply this technique to the study of the morphogenetic changes associated with the process of flowering in the angiosperms, and the results seem to be very promising. The culture of organs of a determinate nature such as leaves will also, I anticipate, be applied extensively in the future to problems of organ determination. What I predict for the future in this area is that we are now, in botanical science, entering into an era of what we might call experimental morphogenesis, somewhat comparable to development of experimental embryology in the animal field many years ago. In the case of plants, I am sure that the entire development of this field will be strictly dependent upon the use of sterile culture techniques, or what we refer to as the tissue-culture technique, although we will obviously not be working with what can be strictly defined as true tissue cultures in every case.

There is another area, not suggested by Dr. Fell's comments, that I introduce now with a certain amount of trepidation, fearing that it is perhaps not pertinent to this discussion. I believe that the use of tissue-culture techniques can be very helpful in the elucidation of what might be termed fundamental morphological problems, of which there are many. A particular example that occurs to me and on which work is now in progress is the old problem of the alternation of generations in the vascular plants, which has been with us since its original discovery by Hofmeister. The prothallial or gametophytic stages of a number of species which have always been difficult to study may now be brought into sterile culture and observed in much more detail than had ever been possible before. An example of this is the cultivation of the gametophytes of *Lycopodium*, which, as many of you know, are extremely rare in nature. Such cultures make it possible to observe certain phases of the life cycle, which are difficult to study otherwise, and they also enable one to test the full potentialities for development of all parts of the life cycle. In a similar vein we are currently carrying on an experimental study of the phenomenon of apogamy in ferns, and I believe that *in vitro* investigations of this sort may have some promise for the future. I should like to mention, in the same category, some survey work of a very interesting nature done in the laboratory of the late Dr. LaRue, in which the potentialities of the male and female gametophytes of certain species of gymnosperms were studied. Work of this sort, which enables us to test the potentialities of structures that normally have a very limited amount of growth and are very much reduced in the life cycle, hold great promise for the future. I should certainly like to see this work extended.

I have simply pointed out a few areas in which plant tissue culture may be likely to expand in the future in its relation to morphogenesis. The selection of areas quite obviously has been governed by the points that Dr. Fell raised, but I think it is of interest that one may make such a comparison between plant and animal fields. Also, they have been governed in part by my own interest. I hope, therefore, that if I have omitted something of great importance or if I have included something that may be trivial, some of those who are more competent to judge than I will comment upon such omissions or unnecessary inclusions.

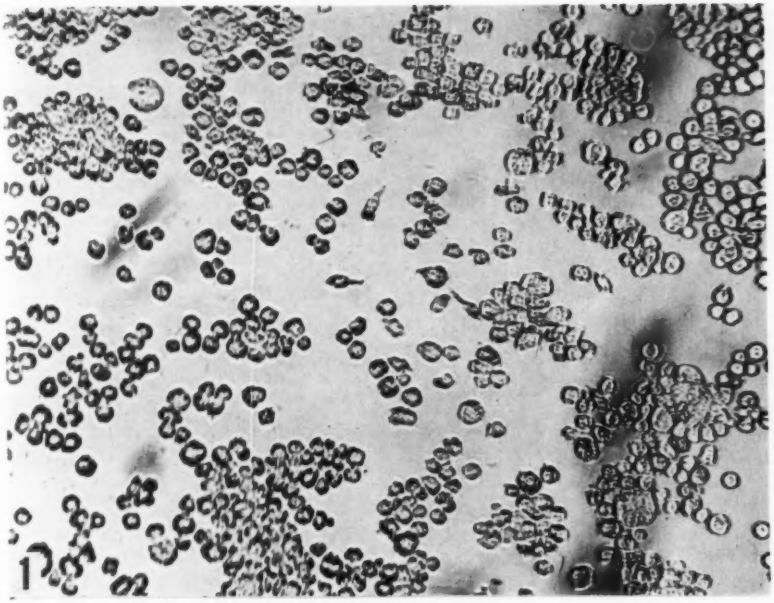
Miss Owens: A strain of mouse lymphoblasts (MBIII), isolated by Dr. deBruyn while working in our laboratory is shown in plate 58. Since its origin many years ago it has been maintained by Mrs. Gey. Figure 1 shows the appearance of the cells with normal calcium concentration. Figure 2 shows cells grown in a medium with greatly reduced calcium concentration. We obtain the reduced calcium concentration by treating the serum and embryo extract with an ion-exchange resin and then adding back, in the form of a simplified balanced salt solution, ions at concentrations that we wish to study. With greatly reduced calcium concentration, we find that we get no reduction in growth rate, but characteristic spindle forms appear. I thought these results might be interesting in connection with the other discussions of fibroblast-like forms.

There are two things to notice in the figures. Most of the cells in the normal medium are round, while those in reduced calcium are spindle-shaped. The cells in the normal medium tend to stick together, while the cells in low calcium always spread out evenly over the surface of the tube.

PLATE 58

FIGURE 1.—Cells of mouse lymphoblasts (MBIII) grown in a nutrient of normal calcium concentration.

FIGURE 2.—The same cells grown in a medium with greatly reduced calcium concentration.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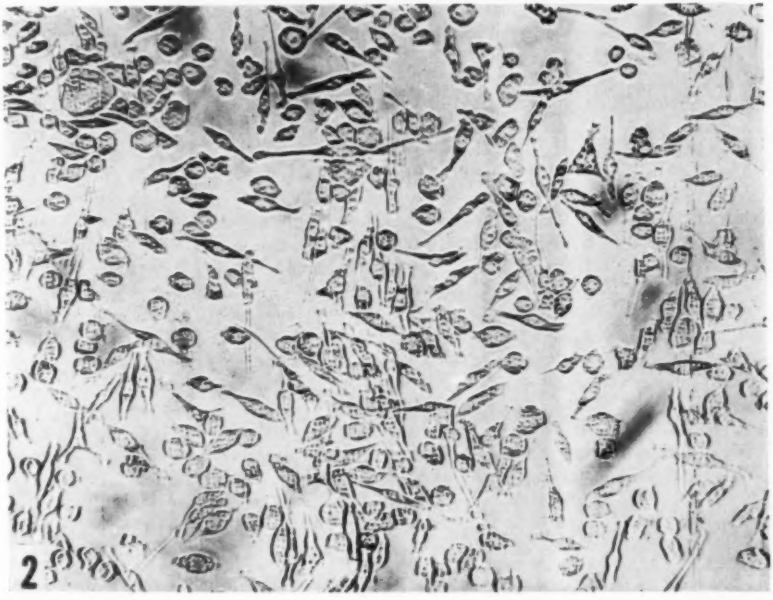
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Dr. Boll: I am very interested in the use of antimetabolites in the study of morphogenesis. I think there is also scope for this kind of work in plant morphogenesis. For instance, the excised tomato root is a relatively simple morphogenetic system. There are really only three features of growth to consider. One is the growth in length of the main axis, the second is the growth of the laterals, and the third is the number of laterals that are produced on the root. There is evidence for an "auxin-type" correlation system within the excised root. In a study of the effects of a number of antimetabolites on this particular system, it was found that some of them influenced the final morphology in quite a profound way. One of the substances that has the most profound effect is ethionine, an antimetabolite for methionine in the excised root. Interpretation of experiments of this kind is rather difficult, especially when there is a correlation system within the root. We are not quite sure what is happening, but the effects may give us some clue to the nature of the auxin system. It was, therefore, of interest to test ethionine on growth systems other than the excised root. This antimetabolite is, in fact, active in a test for auxin activity. Furthermore, it promotes the growth of discs cut from the leaves of etiolated bean plants, provided these discs are given a brief exposure to light.

Dr. Thimann: Since I have never worked in experimental embryology, I hesitate to bring this up, but when one thinks of differentiation and growth of an embryo in biochemical terms, and in terms of the movement of substances, one of the major difficulties is that of the very small distances involved. It is difficult to imagine the maintenance of a biochemical gradient within a meristem or an embryo except under one special condition. That condition is, of course, that the molecules are so large they move with extreme slowness. It was very interesting that Dr. Levi-Montalcini spoke of a protein factor in the differentiation of nerves. I wonder whether future trends in this area might involve the study of the movement of proteins and other large molecules rather than that of small metabolites and known growth substances?

Dr. Gey: Dr. Fell has mentioned the importance of examining an individual cell in great detail both under phase and with the electron microscope. Some years ago, Dr. Bang and I carried out preliminary studies of this type on some of our cultured cells. An extension of one phase of this has been published by Borysko and Sapranauskas. Even more brilliant material from our studies, yet to be published, illustrates quite clearly the preservation, in the final evaluation in the electron microscope, of most of the details of the cellular display originally seen in phase. The techniques used by our group permitted a continuous motion-picture record of the original living phase image through all stages of the fixation with buffered osmium and the completion of the embedding in methacrylate. This was done in a perfusion chamber. The images, on comparison, were remarkably similar when allowance is made for differences in the indices of refraction of the surrounding media. Practically all the structures that one can resolve with phase microscopy are preserved in their exact position, with the exception of some minor displacements. I am of the opinion that the nuclear membrane was more prominent in the fixed specimen. These comparative studies provide much confidence in our evaluation of what may be seen in cells. A further effort is being made to complete the over-all structural concepts with thin sections of cells whose past activities and structural composition are known.

Dr. Grobstein: I am interested in Dr. Thimann's suggestion. I wonder whether in the embryo there aren't materials that actually serve to slow down movement—relative to rates in ordinary solvents—and whether some of the highly specific reactions that take place in very small distances are not made possible by isolating mechanisms that prevent loss of interactants through diffusion. The frequent observation of the jelly-like character of early developmental stages and the possibility that highly polymerized matrix materials in the embryo may be restricting diffusion or affecting its character through differential adsorption is relevant. This may be a mechanism specifically slowing the movement of particular compounds, and "chromatographing" mixtures.

Dr. Steward: My own conviction in getting into tissue culture was not that tissue culture was a way of life but rather that it was the only suitable way for studying

metabolism. This led us to make an intensive investigation of protein synthesis in tissue cultures, using carrot tissue in the presence or absence of coconut milk. This has led to an observation that may have a bearing upon what Professor Thimann had to say.

In every case where cells are in rapid random proliferation, the alcohol-insoluble material, which for this purpose we call protein, is extremely rich in hydroxyproline. This amino acid is one that does not normally turn up in large amounts in plant proteins, though, of course, it is a regular component of animal proteins, particularly of collagen. In one case there was actually a protein in which about 8 percent of the total weight was represented by hydroxyproline. This led us to see whether enrichment with hydroxyproline was general. We used cells of carrot grown in culture, potato tissue grown with the aid of 2,4-dichlorophenoxyacetic acid (2,4-D), Jerusalem-artichoke tissue, crown-gall tissue, and a number of other similar cultures. In every case that we have examined, the protein, or at least the alcohol-insoluble material containing the protein, shows a strange enrichment in hydroxyproline. Therefore I wonder whether, in some of these situations we have been thinking about, we have really fully explored the possibilities of identifying the actual biochemical constituents characteristic of this kind of material. Whether these are causal agents of division or not is another matter, but it does seem to me that there is an important area where the characteristics of the dividing tissue can be examined by a biochemist.

Looking into the long and distant future, I have always thought I would like to live to see the day when somebody would really tell me what the difference is within a meristem cell in a root apex and the meristem cell in the shoot apex. When I was fortunate enough to teach these subjects, I was always impressed by the fact that one cannot see any difference. If one could actually explant some of the material from one apex, put it in the other, and see what happens, it seems to me this would be extremely illuminating.

I have always been impressed by the fact that there are some familiar structures that are still very baffling. I never quite know why the Katahdin potato, one of the horribly tasteless potatoes that have been developed in the United States, should ever produce this massive structure in the first place. What the coconut milk does to the carrot, it seems to me, the potato leaf does to the stolon. How can we regard the growth of some of these vegetables, such as carrots, artichokes, and potatoes? Is a potato tuber the equivalent of a tumor on *Solanum tuberosum*? Thus it seems to me that there are a lot of rather simple observations that can be made and that still need morphological explanation, and I foresee the day when the techniques of experimental morphology through tissue culture, correlated with some of the more biochemical observations I have indicated, may bridge the gap between photoperiodic and other phenomena and the ultimate causal effects of the environment. It does seem to me that there is a bright future for those who will combine the morphological approach with the attempt to express these problems in terms of real biochemistry.

I do feel that only the edge of the problem has been touched, because very few people have done intensive biochemical examination, by chromatographic or other means, of the differences in the different kinds of structures in relation to their ability to grow. I would make a plea for the use of the tissue-culture technique particularly in this area where a great deal is to be learned by the methods that we already have.

Dr. Wilde: I want to rise to the defense of the experimental use of small diffusible molecules partly because, as an "embryo chemist" as well as a chemical embryologist, I feel more at home with them. They are easily handled quantitatively and are distinctly characterized chemically. These factors help when one is dealing with living protoplasm. Within protoplasm are so many complex protein and other high molecular weight constituents that one can easily overemphasize certain particular ones and neglect others. Another point, in defense of the experimental use of the diffusible molecule, is that often we begin with the input into a living system and then try to get some register of an output system or identifiable end product. We may have, as yet, very little information with regard to what goes on in between. This is particularly true when we are dealing with the determination of morphology.

Finally, we may sell protoplasm short if we do not consider that there may be a selection mechanism within it that will pick out the desired diffusible molecules and reject others.

Dr. Potter: I think that there is a great deal to be said for the small diffusible molecule, but I foresee that tissue culture in the future will devote increased attention to the nondiffusible molecule. This includes the effects of ribonucleoproteins and such molecules on the cultures. Here I want to point out that there are a number of biochemists who are mechanically tearing cells apart in getting out these ribonucleoproteins. The only tests that we have as to whether these compounds are suitable for you tissue culturists to use is to test them and to see what their properties are and to correlate those properties with the properties that they exhibit in the living animal. Let's give particular attention to the condition of these ribonucleoproteins with which we treat the tissue culture. I would like to suggest an experiment that I am not in a position to do myself. This experiment is based on what I feel is a very probable situation, namely, that these cells which are undergoing differentiation and losing the power to divide, have lost a particular kind of ribonucleoprotein that is necessary for the synthesis of deoxyribonucleic acid. If this is true, one should be able to take a culture consisting of differentiated cells that cannot divide and add to it ribonucleoprotein particles from cells that can divide and try to induce the power of cell division in those highly differentiated cells. Some day, I think it can be done.

Viruses and Other Pathogenic Agents in Plant Tissue Cultures [1, 2]

L. M. BLACK, *University of Illinois, Urbana, Illinois*

My assignment for this symposium, as indicated by the title, requires the consideration of diverse biological materials. I shall attempt to deal with these under the following topics: tissue culture of crown gall and a possible causal virus, tissue culture of plant tumors incited by wound-tumor virus, other viruses in plant tissue culture and, finally, tissue culture and nonviral parasites. I shall not make an attempt to include all the information extant on these various topics; I have endeavored to focus attention on unresolved problems, on implicit assumptions, and on future possibilities.

Tissue Culture of Crown Gall and a Possible Causal Virus

Although the problem of crown gall in relation to plant tissue culture will be discussed in a later session, it would seem appropriate here to raise certain etiological questions regarding this disease, especially those concerned with the possible causal role of a virus.

The crown-gall problem is still one of intense interest to plant pathologists and cannot fail to interest biologists in general. I am glad that I am not expected to review the vast literature that has accumulated on this disease. This task has been performed very competently by Riker and Berge (*1*), de Ropp (*2*), Braun (*3*), and Klein and Link (*4*) in contributions that are very valuable and informative for those of us who are not working directly on this disease.

We have long been familiar with the role of the crown-gall bacterium in inciting this disease and this familiarity may at times have caused us to forget the magnitude of the advance made when, in 1906, Smith and Townsend (*5*) discovered that they could induce crown gall by inoculating plants with pure cultures of a specific bacterium. We are also indebted to Smith, in my opinion, for his boldness in homologizing crown gall with cancer in animals.

The convincing demonstration by Braun and White (*6*) that crown-gall cells are autonomous tumor cells, capable of continued growth as tumor

[1] Received for publication May 28, 1957.
[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8–12, 1956.

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cells in the absence of the inciting bacteria, was a tremendous forward step and has centered our attention in this disease on the problem of what emanates from the bacteria to convert healthy cells into tumor cells and what maintains these cells as tumor cells. The factor that mediates the action of the bacteria on the cells has been designated "the tumor-inducing principle (TIP)" and there have been suggestions that this tumor-inducing agent may be a virus (*2*, *7*, *8*).

Perhaps the experiments most suggestive of virus involvement are those tissue-culture experiments of de Ropp (*7*, *9*, *10*) demonstrating that when bacteria-free crown-gall tissues of sunflower are grafted onto normal sunflower stem segments *in vitro*, tumors may arise from the previously healthy tissue. These will be referred to as "appositional" tumors (*4*). Such tumors are capable of profuse growth on media in which growth of normal sunflower tissue is not sustained. These observations have been confirmed by similar experiments by Camus and Gautheret (*11*) and by McEwen (*12*).

de Ropp's experiments demonstrated the transfer of a tumor-inducing agent from crown-gall cells to healthy cells in the absence of cellular organisms—experimental results which could be accounted for by a virus. If one considers all the unlikely places and unexpected effects wherein viruses have been demonstrated to be the significant etiological factor, he will feel a responsibility for not abandoning the virus hypothesis lightly, because of any difficulties of an inconclusive nature it may encounter. With this responsibility in mind, I should like to re-examine some of the facts that seem at variance with the virus hypothesis. I hope I may do so without being typed as one who "believes" that the tumor-inducing principle is a virus.

In their excellent review, Klein and Link (*4*, p. 260) have listed some of the facts that seem inconsistent with the virus hypothesis. Many of these points have also been raised by others, but Klein and Link's discussion is perhaps the most comprehensive. While they do not say or imply that these facts eliminate virus as the tumor-inducing agent, it may be worthwhile to consider them from a different point of view.

The various failures to obtain tumors by inoculating plants with preparations of crown-gall bacteria lysed by phage in order to liberate a hypothetical virus (*4*, p. 260) do not constitute critical tests demonstrating the absence of such a virus. Many plant viruses, *e.g.*, aster yellows and potato leaf roll viruses, cannot be transmitted to plants by such methods of inoculation. Moreover, the phage might have destroyed the virus or the plant cells might not have been in a susceptible condition. The failure of White and Braun (*13*) and of Klein and Klein (*14*) to transmit a tumor-inducing agent to healthy plant cells or to avirulent bacteria, respectively, by inoculating them with extracts of bacteria-free tumor cells, cannot be regarded as critical evidence against the virus hypothesis for the same reason, namely, the failure, thus far, to transmit many known plant viruses to susceptible hosts by such techniques.

Suppression of tumor formation by the application of antibiotics to

inoculated tissues also cannot be considered as an argument against the virus hypothesis. Although the antibiotics used in such experiments were not antiviral, they might have interfered with the role of the bacteria as vectors of a virus. They might also have affected directly the tumor cells themselves.

The absence of virus inclusion bodies in cells of crown galls can scarcely be regarded as evidence against the virus hypothesis, because a number of plant viruses have been observed not to produce inclusions in the plant cells they infect. Nor can the failure (*14*) to demonstrate a lysogenic virus in crown-gall bacteria be considered as evidence against the virus hypothesis. A hypothetical virus having the properties of the tumor-inducing principle need not have the property of lysing bacteria in the manner that makes possible the detection of lysogenic phages.

Although the appositional tumors in de Ropp's tissue-culture grafting experiments have, at first, an organization different and more nearly normal than that of primary crown-gall tumors, and although bacteria are not present at their induction, these criteria would seem insufficient basis for concluding that the tumefacient agent is different in the two cases (*4*, p. 234). On passage, de Ropp's appositional tumors assume the same structure as primary tumors. It has been demonstrated by Braun (*15*) that the same inoculum may produce, from cells derived from the same site on the plant, tumors of either the completely unorganized or the teratoma type. In this instance, factors determining tumor type were the growth of the tumor on the plant, rather than in culture, and the polarity induced in the part inoculated. The same considerations are pertinent to an evaluation of the assumption that the agent which induces secondary tumors on inoculated plants is different from the agent, coming from bacteria, that induces primary tumors (*4*, pp. 232 and 234).

It has also been suggested that the agent maintaining the tumor cells, as such, is different from the agent inducing the tumor cells, because the former survives in cells maintained at 47° C. for long periods whereas the latter is inactivated around 30 to 32° C. (*4*, p. 261). However, it is known that viruses may be much more stable to heat inside the plant host cells than they are in an entirely different environment outside the cell (*16*).

Many of the experiments demonstrating the facts discussed above were ingenious in conception. Nevertheless, it is apparent that many of these facts are not contrary to the existence of a causal virus and failures to demonstrate virus cannot be regarded as eliminating virus as a possible causal agent. Positive findings demonstrating the nature of the tumor-inducing principle as some agent other than virus would eliminate virus as a possible cause.

We do not know of any virus that is transmitted to plants by a bacterium. If the tumor-inducing agent in crown gall is a virus, then its maintenance in bacterial cultures through many transfers would require its multiplication in the bacteria. It is pertinent to recall the established fact that some viruses multiply in both plants and insects (*17*, *18*), that hog influenza virus is maintained in lung worms through a complicated

life cycle in pigs and earthworms (*19*) and that a rickettsial organism causing a serious disease in dogs is maintained in flukes undergoing an involved life cycle in snails and salmonid fish (*20*). It seems probable that both the influenza virus and the rickettsia are maintained by growth in their helminth carriers.

There are certain facts about crown gall that are particularly suggestive of virus, apart from those basic facts already mentioned, such as the passage of some tumor-inducing agent from the bacteria to the plant cell, and de Ropp's demonstration of the passage of such an agent from tumor cells to healthy cells without the mediation of bacteria.

An observation reported by Nickell (*21*, p. 117) is also of interest in connection with the virus hypothesis. A crown-gall tissue of sweet clover clone C_{10} was cultured free of bacteria for some months when a group of subcultures suddenly formed roots over their entire surfaces. Some of these roots were subcultured and grew well as roots for 14 months. Then, without apparent cause, the roots in several flasks started forming tumors that looked like the original crown gall. Bouriquet (*21*, p. 121) has observed a similar phenomenon in tissue cultures of crown gall of *Scorzonera*. Démétriadés (*21*, p. 121) has suggested that a carcinogenic principle remains latent in the root during the nontumorous phase. The latency of some factor, indicated by these observations, is reminiscent of the latency of bacteriophages in lysogeny. It has been found that the latency in such phage infections of bacteria can be broken by certain irradiations and by certain substances (*22*). It would be interesting to investigate the effect of similar treatments of the above roots or of other organs apparently carrying a latent factor for tumorogenesis.

Preliminary reports of a high-molecular-weight protein constituting as much as 20 percent of the protein content and occurring in crown gall of several species but not in normal tissue or habituated tissue (*23–25*) is also consistent with a virus etiology. Failure to produce infection with this component is not inconsistent with its being a virus. As pointed out before, many viruses have not been transmitted to plants mechanically. It is important to know the final outcome of these studies. Braun (*26*) reported a slight but apparently specific precipitin reaction for an antigen present in crown-gall tumor tissue and absent in healthy tissue of the same plant species. However, his precipitin tests failed to reveal the same antigen in crown-gall tissue from distantly related plants. It should be remembered in this connection that serological reactions have been successful thus far for only a small minority of plant viruses.

It might also be pointed out that the very interesting work of Braun (*27*), demonstrating the recovery of crown-gall teratomas under forced growth, is in harmony with virus participation in the etiology of crown gall and Braun's hypothesis of self-replicating cytoplasmic particles is not inconsistent with it. The same is true of the work of Van Lanen, Baldwin, Riker (*28*) and others showing that crown-gall bacteria may

become avirulent when grown through several passages in a medium containing glycine or certain other amino acids. Both kinds of experiments may be interpreted as indicating the loss of virus from its host cells under conditions unfavorable for its reproduction at a rate sufficient for maintenance.

Finally, the high energy of activation, 80,000 calories per mole (*29*) for the destruction of the tumor-inducing agent at the inoculation site, is consistent with the hypothesis that it may be a virus.

Klein (*30*) and Klein and Link (*4*) have suggested, on the basis of a number of experiments, that the tumor-inducing agent may be nucleic acid. It is to be hoped that at this symposium the status of Klein's most interesting studies will be brought up to date, and, in particular, that we will ascertain whether he and others have been able to confirm his reports of production of primary tumors with bacteria-free filtrates and the transformation of avirulent *Agrobacterium tumefaciens* and even nontumefacient, related, bacterial species into virulent crown-gall producing organisms. In connection with these studies on the possible role of nucleic acid, it is worth remembering that Hershey and Chase (*31*), Hershey (*32*), Gierer and Schramm (*33*), and Fraenkel-Conrat (*34*) have demonstrated that viral nucleic acid is the virus constituent essential to its replication in the host cell and probably the only viral constituent indispensible to virus reproduction. It is therefore possible that nucleic acid preparations from either tumor cells or virulent bacteria, if capable of inducing typical crown gall, might owe their activity to nucleic acid from a virus.

These facets of the problem are tantalizing to a virologist because, of course, to be satisfied of virus participation, one needs conclusive evidence of an infective virus particle and not mere consistency with a virus hypothesis. Although the author is not committed to the idea that a virus is the tumor-inducing agent in crown gall, it has seemed worthwhile to reconsider, in the light of our general knowledge of viruses, points that have been advanced against the virus hypothesis. This hypothesis, it seems to me, is still apposite and it owes its principal supporting data to studies with tissue cultures.

Before leaving the virus hypothesis of crown gall, it is worth recalling one feature of the disease that presents unusual difficulties to its acceptance. Braun (*35*) demonstrated that tumors with a proliferative vigor, ranging from very poor to full, were obtained by allowing the bacteria to act on susceptible *Vinca rosea* L. plants for periods varying from 2 to 5 days. These tumors maintained their characteristic degrees of proliferative ability in tissue culture. One would expect a successful virus infection to produce the same result no matter how long the exposure. One would also expect that if the graded series of tumors were the result of varying mixtures of fully tumorous cells and normal cells, that the former would eventually eliminate the latter on continued growth in culture. Perhaps this is not so, and a stable mixture is in some way maintained. There are plant virus infections in which the quantity of inoculum—tomato

spotted wilt—or the site of inoculation—curly top (*36*)—has an important influence on the severity of systemic infection, but it is difficult to see how this would occur in individual cells. To account for different responses by individual cells, it would seem necessary to postulate local limited infections of organelles within the cell. The only basis for such virus states, of which I know, is the condition obtaining in lysogenic bacteria.

Tissue Culture of Plant Tumors Incited by Wound-Tumor Virus

In order to comment on certain problems connected with the culture of tumors caused by this virus, I think it appropriate to outline here, in more detail than has yet been published, the circumstances of the isolation and culture of such tumors on roots of *Rumex acetosa*.

From September 21 to October 3, 1944, attempts were made to isolate tumor tissue incited in various plants by wound-tumor virus and to grow them on a sterile medium. The media employed in this endeavor and in subsequent transfers were either White's basic agar medium (*37*) or slight modifications of it. Niacin and yeast extract were incorporated in most preparations and, to some, an extract of sorrel leaves was added. The latter ingredient did not make an appreciable difference in growth and after a 12-month trial, it was omitted.

The neoplastic wound-tumor tissues from which isolations were attempted were taken from plants of *Anagallis linifolia* L., *Chrysanthemum leucanthemum* L. var. *pinnatifidum* Lecoq and Lamotte., *Linum grandiflorum* Desf., *Melilotus alba* Desr., *Nierembergia frutescens* Dur., *Reseda odorata* L., *Rumex acetosa* L., *R. obtusifolius* L., *Silene latifolia* (Mill.) Britten and Rendle, and *Vinca rosea* L. The only tissue that grew well was tumor tissue from roots of *Rumex acetosa*, and efforts were soon focused on it alone. Tissue from the crowns of healthy sorrel plants did not grow on the same medium. Subsequently, the cultured sorrel-root tumor tissue was given the abbreviation R_1 to indicate that it represented the first isolate of wound-tumor tissue from *Rumex*. Not enough work was done with the other tissues to determine whether they could or could not be grown with the methods used. Plant hormones were not employed.

The sorrel tumor cultures were transferred at various intervals and increased sufficiently so that the better clones could be transferred and the poorer ones discarded. Sterility tests of tissue, ground tissue, and exhausted medium were made in Difco-Bacto nutrient broth on June 14, July 6, and August 16, 1945. No clones were proved to be contaminated. However, in a few cases, some of the test tubes of broth showed growth of organisms after being kept for many days. Tumor lines providing inoculum for such tubes were discarded. Nickell (*21*, p. 120) has made much more elaborate and conclusive tests demonstrating the absence of cellular organisms in this tissue.

The question of detection of virus in the tissue has been discussed recently (*21*, pp. 120–121), and it seems appropriate to comment in detail on this problem at the present time.

Inclusions occur in tumors incited by this disease, but because inclusions may occur in plants as a result of factors other than virus and because those associated with wound-tumor disease have not been demonstrated to be specific for virus infection, their presence in cultured tissue, although indicative, cannot be considered conclusive evidence of virus infection.

At first, the only methods known for transmission of wound-tumor virus were by grafting and by the specific insect vector, after acquisition of the virus by feeding. The virus has since been transmitted through juice from sweet-clover tumors by pinpricks into the crowns of young crimson-clover plants. However, transmission by pinpricking juice from diseased plants into healthy plants gives only poor results even when a rich source of virus is employed (*38*). The virus has never been transmitted by rubbing juice on susceptible leaves. An insect vector may be unable to acquire virus by feeding, or be able to recover it very poorly, from certain susceptible plant species (*39*, *40*). Although the field strain of wound-tumor virus can also be transmitted by injection into leafhoppers, which are subsequently tested on susceptible plants, the virus might undergo changes during its persistence in cultured tissue. In view of recent work on vectorless plant viruses (*41*), it is even probable that the virus, if still present in the R_1 tumor tissue, has become vectorless during its long reproduction in sorrel tissue without passage through the insect. In other words, it is probable that it has lost its ability to be transmitted by insects whether it is introduced into the vector by feeding or injection. Failure to detect virus in a tissue by juice inoculation or by tests using the vector is therefore of no significance in indicating that a virus is absent in the tissue. Some of these difficulties of demonstrating the presence of a known virus might well be borne in mind when considering failures to demonstrate virus in a disease like crown gall.

On the other hand, a test by graft transmission is, except in the case of viruses that produce only local lesions in the tissue to be tested, an extremely sensitive test for virus. In fact, I know of no more sensitive test for a plant virus, provided the tissue suspected of harboring virus survives at the graft union and conditions are favorable for virus invasion and symptom expression in the stock.

On November 29, 1945, tests for virus in the R_1 tissue were made by grafting the cultured tissue to young healthy sorrel plants. The stem of *R. acetosa* is very short and bears only a rosette of leaves until late in its development; it was considered unsuitable as a site for grafts. Young plants were transplanted several times in such a way as to raise the crown higher above the surface of the soil on each occasion, and the exposed root was reduced to a single trunk by pruning. Slices of cultured sorrel tumor tissue, previously shown to be free of bacteria, were inserted into longitudinal slits through this root trunk and wrapped with "Sterilastic," an elastic rubbery tape that adheres to itself. Sixty days after grafting, 1 grafted plant showed a tumor at the graft site but no other symptoms. Seventy days after grafting, all the leaves were removed from each plant except 1 young leaf about an inch long near the growing point. Fourteen

weeks after grafting, tumors had developed at only 2 graft sites out of 15 grafted plants, but both plants showed severe leaf symptoms of wound-tumor disease and 1 showed root tumors. None of the 13 other grafted plants and none of 15 control plants showed symptoms of wound-tumor disease. Plants of *R. acetosa* are extremely variable in reaction to wound-tumor disease. Some healthy plants show an irregular enlargement of the veins that is difficult to distinguish from mild vein symptoms of the disease. Because of the sequence of symptoms in the 2 diseased plants, the absence of any disease in 15 control plants, and the origin of the culture, I concluded that wound-tumor virus was present in the tissue (*42*). This conclusion is not so well grounded in experimental evidence as it should be, and, as far as the author is aware, no other tests for the wound-tumor virus in the R_1 tissue have been reported.

There is, however, no reason to doubt the presence of the wound-tumor virus in the original R_1 tissue. The plant from which the tumor tissue came was infected with the virus. The virus content of sorrel tumors has not been successfully measured, because juice from sorrel plants effectively precipitates proteins. However, the virus content of tumors on sweet clover infected with the same virus is roughly 100 times that of nontumorous parts of the plant. Both sorrel and sweet-clover tumors are rich in inclusion bodies.

Although the wound-tumor virus was present in the R_1 tissue orginally, and apparently at the time of the above test, it is possible that it is no longer present. It is worth recalling that the Shope papilloma virus was lost from the V_2 carcinoma, of which it was an incitant, after successfully maintaining itself in the tissue for many passages (*43*). Since many experimental results with the R_1 tissue are interpreted on the assumption that the virus is still present, we have recently undertaken to test again for its presence. Graft-transmission tests are under way.[3] Recently, we (*44*) have obtained an antiserum of high titer for the wound-tumor virus. This antiserum failed to reveal any viral antigen in extracts of tissue cultures. It cannot be concluded from these grafting or serological tests that the virus is not now present in the R_1 tissue. It should be apparent from this discussion that whereas positive results by any of the tests discussed can be significant, negative results are not significant except in the case of the test by successful grafting of scions.

With this description of the origin of the cultured R_1 sorrel tumor tissue in mind, let us now consider some researches conducted with it. There is no need for me to review the researches that have been done on cultures of wound-tumor sorrel tissue (R_1). This has been done very adequately by Brakke and Nickell (*21*, *45*). It is necessary, however, to my discussion to mention some salient results of this research.

When Nickell began his work with the R_1 tissue, the only points that

[3] The tests were carried out between June 15, 1956, and March 8, 1957. Scion tumor tissue consisting of thin slices of the cultured tissue was grafted to 50 sorrel plants in the manner described above. Forty-eight ungrafted plants were retained as controls. None of the scion tumor slices grew. In most cases the tops and roots of the grafted plants were severely pruned back 11 weeks or more after grafting and the plants observed for an additional 9 weeks or more. At the end of observation periods of 16 to 38 weeks from the time of grafting, 42 grafted plants and 41 control plants survived. None showed symptoms of wound-tumor disease in roots or tops.

had been demonstrated were its ability to grow in culture, probably indefinitely, as tumor tissue containing the inciting virus but free of cellular organisms. Some of the most interesting research results by Nickell, Brakke, and others are the demonstrations that this tissue has an optimum phosphate requirement 10 to 100 times that common for other tissues (*21*, p. 108), that ribonucleic acid (RNA) hydrolysates are stimulatory, and that deoxyribonucleic acid (DNA) hydrolysates are slightly inhibitory. The release from living tissue cells of an enzyme, α-amylase, is of considerable intrinsic physiological interest. It seems probable that this enzyme is a protein that is secreted through plasma membrane and cell wall, although it is possible, as suggested by Steward (*45*, p. 225), that it may be elaborated at the surface of the cell. The description of various biochemical reactions of the tumor has proceeded rapidly, though not always with a precise identification of the tumor tissue or virus involved. This could be accomplished by indicating that the tissue is the R_1 isolate from *Rumex acetosa*. There might easily be other isolates of tumors caused by the same virus in *Rumex*. Also, it is not always clear that the tissue was free of contaminating organisms at the beginning and the end of biochemical or physiological experiments. Since Robbins and Hervey (*46*) pointed out the frequency with which plant tissue cultures may be contaminated, precautions such as those described by Nickell (*21*, p. 120) are essential.

One of the interesting possibilities provided by the culture of neoplasms incited by wound-tumor virus is that of disclosing how the virus induces tumor growth. However, the R_1 tissue is, at present, liable to several deficiencies for this purpose. Other than cultured sorrel roots, there is, at present, no control culture of *Rumex acetosa* known to be free of the virus. Certain differences between the tumor and the roots might be due to the very different number of meristematic centers. The ideal control culture would be a virus-free tumor type or callus-type tissue isolated from the same clone at the same time as the isolation of tumor tissue induced by virus. Plants grown from seed of *R. acetosa* are extremely variable morphologically and probably differ physiologically. Clonal reproduction of certain individuals of *R. acetosa* would be an advantage in studies of this disease, but we have found it difficult to obtain and maintain such clones. It is apparent from this discussion of the history of the R_1 isolate that during the maintenance of this tissue in culture, selection of tissue variants and virus variants was possible. Thus, the longer the interval between the isolation of the virus tumor and the isolation of the control tissue, the greater the opportunity, it would seem, for factors other than the original virus to account for any difference between tumor tissue and control tissue.

Nickell (*21*) has attempted to obtain cultures of crown-gall tissue and habituated tissue of *Rumex acetosa*, but thus far without success. Nickell's progress in isolating neoplasms induced by wound tumor, crown gall, and indoleacetic acid from the C_{10} clone of sweet clover is a step in the right direction and has already provided additional evidence (*21*, p. 116) that

the unusually high phosphate requirement is associated with the wound-tumor virus.

Is the high phosphate requirement, apparently associated with this tissue, necessary because it contains a plant virus or because the virus it contains is tumefacient? The same question might well be applied to the reaction of the R_1 tumor to RNA hydrolysates, nucleic acid bases, and their analogues. Is the release of α-amylase a property of *Rumex acetosa* tissue, of such tissue infected by virus, or of such tissue infected specifically by the wound-tumor virus? The sorrel roots grown *in vitro* are not a sufficiently close control on the cultivated tumor tissue so that one can be certain of the answers.

What biological resources do we have that might be brought to bear on these questions?

First of all, it should be pointed out that the 50-odd plants first reported as susceptible to this virus were disclosed by a preliminary survey of about 100 plant species. The tests were not rigorous enough to prove that the species which were not infected were immune. It is probable that any other 100 species similarly chosen would yield about as many suscepts if similarly tested. No systematic survey of species related to known suscepts has been made.

Other viruses are known to infect *Rumex* sp. and could be used as controls on the tumefacient character of the wound-tumor virus.

It is also certain that there are other tumefacient plant viruses such as the virus causing Valleau's club root of tobacco (*47*). The vein enlargements in the crinkle disease of *Crotalaria* in Indonesia (*48*) remind one of wound-tumor disease in certain plants. We have recently found a slightly, but definitely, milder strain of the wound-tumor virus under circumstances which suggest that if the virus is still present in the R_1 tissue, it is probably less virulent than it was originally. Comparisons of pathogen strains of different tumefacient ability have been illuminating in crown gall and might also be so with wound tumor.

The wound-tumor disease has been known for a much shorter time than crown gall and has been studied much less. There is still a great deal to be learned about it.

Other Viruses in Plant Tissue Cultures

Before the isolation of sorrel-root tumor tissue in culture, in fact, as early as 1934, White isolated and grew, in culture, roots from infected tomato plants. This enabled him to study the distribution of tobacco mosaic virus in tomato roots. Morel (*49*) has employed essentially the same technique of isolating infected plant parts and in this way, tobacco mosaic virus, potato X virus, and potato Y virus have been obtained in cultured tissue. One of Morel's most successful techniques was to isolate crown-gall tissue from plants infected with the desired virus. Studies on such tissues have been designed to determine the effect of various environmental conditions or substances in the medium upon growth of the tissue and upon the virus concentration (*50–52*). Aseptic sections of infected

tobacco stems induced to form callus in culture have been used in attempts to find antiviral substances (*53*, *54*). In some cases wet weights have been employed to measure growth and sometimes reduction in virus concentration is inversely proportional to growth, so that it is uncertain to what extent the lower concentration might be caused by dilution.

Difficulties have been encountered in inoculating virus into healthy plant tissues in culture. Morel inoculated virus, by several methods, into previously virus-free crown-gall tissue growing in culture. His evidence for success is most convincing where he used the less stable potato Y virus and still found it present after 2 transfers during a 4-month interval. Ségrétain (*55*) grafted virus-free tobacco crown-gall tissue from culture onto an infected tobacco plant and then reisolated the tissue in culture after it had become infected. This inoculation technique might have advantages in work with very unstable plant viruses.

Morel (*49*) and Ségrétain (*56*) obtained evidence that viruses in cultured tissues were present at much lower concentration than in the leaves of intact plants. Morel suggested that this may be due to the meristematic nature of tumorous tissue because of considerable evidence that in intact plants the virus is in lower concentration in the apical meristem than in differentiated tissue. Because most plant viruses reach their highest concentration in plants that are otherwise vigorous and healthy, it is also possible that the low concentration in plant tissue cultures is due to the impoverished state of such tissues. To a pathologist, cells in plant tissue culture, with their thin walls and sparse protoplasm, have a diseased appearance even though not infected. This, of course, does not mean that they cannot be useful. Possibly more emphasis could be given to obtaining plant tissues in culture growing as vigorously as the tissues in the intact plant, instead of being satisfied with tissue that grows on the optimum concentrations of the minimum number of ingredients.

"One-step growth experiments" with bacteriophage (*57*) and their application to many fundamental bacterial virus problems were not long in being appreciated by workers in other virus fields. It is obvious that if the same techniques can be applied in studies of plant viruses, progress will be greatly facilitated. One-step growth experiments have been carried out with certain animal viruses in tissue culture (*58*) but so far as I know little progress has been made in applying this technique to plant viruses.

The reasons for this meager progress are not difficult to discover. The host cells of bacteriophage grow separately and the bacterial virus has its own mechanisms for being absorbed to the bacterium, penetrating into it, and being released from it. Certain animal cells can be grown as discrete elements. Virus can be absorbed to animal cells, penetrate into them, and later be released from them. In systems of plant cells and virus, none of these important features is naturally present. Except in rare cases the cells grow cemented to one another, and viruses not already inside the cells apparently must be introduced and released through a wound made by some external agent. A general solution to this plant-virus problem of performing one-step growth experiments or experiments

with single plant cells must therefore overcome or circumvent obstacles, most of which are not commonly present in the two other virus fields.

A solution for a special group of viruses that grow in both insect and plant tissue (*17*, *18*) may materialize by growing them in the insect tissues. This may obviate the disadvantages of plant cells for certain studies with these viruses. A start has been made with such cultures (*59*).

Plant tissue cultures have been started from single cells by ingenious techniques (*60*) and, in a particular case, cultures of the cells of a higher plant in small groups or even single elements have been reported (*61*).[4]

A few workers, most recently Benda (*62*), have succeeded in inoculating single cells in their natural position on the plant by micromethods.

Streisinger (*63*) has carried out some preliminary experiments designed to grow plant viruses in cultured discrete cells of higher plants. He reports that it was not difficult to obtain cultures of single cells or clumps of a very few cells in suspension in liquid. These were obtained by growing a friable strain of callus culture of *Nicotiana tabacum* (from Hildebrandt and Riker) or of *N. glutinosa* (isolated by Streisinger) in shaker cultures or in test tubes supplied with air through bubbler tubes. The cells stayed alive on agar plates or in hanging drops for a few weeks, as determined by observations of cytoplasmic streaming or cell division. However, Streisinger did not succeed in infecting such cells with either tobacco necrosis or tobacco mosaic virus. He was also unable to remove cell walls with pectinase.

Steere (*64*, p. 205) has speculated on the possibility of using the plaque technique for the study of plant viruses on tissue cultures of susceptible plant host cells, particularly cells from hosts producing local or primary lesions as a result of inoculation. His discussion contains a number of suggestions for attempting this method.

In efforts to obtain part of the uniformity, reproducibility, and precisely controlled environmental conditions possible with tissue cultures, several workers have used the method of floating detached leaves or leaf discs on nutrient solutions (*65–67*). Although this is not a true tissue-culture method, it does provide some of the advantages inherent in tissue-culture techniques.

Tissue Culture and Nonviral Parasites

The use of true tissue cultures of plant hosts for various studies of their parasites was preceded by the experimental use of entire plants or detached roots grown aseptically, or of leaves or leaf discs maintained by floating them on solutions of sugar and mineral salts. As an example of the use of entire plants, one may cite Chupp's (*68*) experiments, in 1917, with *Plasmodiophora brassicae*. Although there is abundant evidence that this parasite is constantly associated with club root of crucifers, Koch's rules of proof of pathogenicity have never been satisfied for this organism because it is an obligate parasite. The closest approximation to such proof was provided by Chupp who grew cabbage seedlings on sterile

[4] Since this paper was presented, Nickell (*72*) has reported the continuous cultivation of tissues of a higher plant as single cells.

media and inoculated them with the spores of *P. brassicae* in the absence of other organisms. Some other early work of this type has been reviewed by Morel and Gautheret (*8*).

Much work has been done with the floating detached leaf or leaf disc method reviewed by Yarwood (*65*).

The use of true plant tissue cultures in the study of diseases involving cellular parasites other than crown-gall bacteria has been extremely limited.[5] The most extensive work has been done by Morel (*49*) who held that the study of obligate parasites on intact plants or even on detached leaves was subject to a number of disadvantages which he enumerated. Accordingly, he attempted the culture of a number of obligate parasites on cultured tissue of their hosts and succeeded with *Plasmopara viticola*, a phycomycete, and *Uncinula necator*, an ascomycete. He failed to culture any of 4 species of Uredinales, basidiomycetes, but, on the basis of his failures, suggested methods that he thought should prove successful. The techniques of isolation, inoculation, and transfer, described by Morel, should be helpful to any who attempt to carry this work further.

In general, Morel found that both *Plasmopara viticola* and *Uncinula necator* retained their normal host relationship in cultured tissue. *Plasmopara viticola* produced an intercellular mycelium with haustoria and *Uncinula necator* developed a superficial mycelium that sent haustoria into only the superficial layer of cells. However, *Plasmopara viticola* also produced an aerial mycelium not observed in nature. The parasites grew much less vigorously on the cultured tissue than they ordinarily grow on intact plants, perhaps because cultured tissue is not so healthy and vigorous as the tissues of intact plants usually are, and obligate parasites commonly develop most profusely on susceptible plants that are otherwise most healthy and vigorous. Although the parasite grew more poorly the longer the host tissue had been maintained in culture, these less robust combinations seemed to survive longer than those involving recently isolated tissue. The relative susceptibility of different hosts was maintained in culture.

Morel failed to obtain any growth of any obligate fungus parasite on medium in the absence of living host cells. However, Cutter (*69*) and Hotson and Cutter (*70*) claimed to have grown 4 isolates of the rust fungus, *Gymnosporangium juniperi-virginianae* (Uredinales), in a relatively simple medium in the absence of living cells of either of its alternate hosts. There seems to be no doubt that Hotson and Cutter succeeded in growing this rust fungus in cultured host tissue. Hotson (*71*) reported maintaining subcultures, isolated in 1949, for several years. However, the significance of the reported growth of *G. juniperi-virginianae* in the absence of host cells is uncertain. Out of an unstated number of isolates, only 4 are reported to have grown in this manner. The cultured mycelium

[5] Although the *Review of Applied Mycology*, the most complete abstracting journal used by plant pathologists, has carried abstracts of articles on tissue-culture studies under other headings in its indices, "tissue culture" has not appeared as a primary heading during the past dozen years.

was inoculated onto 12 callus tissues of *Crataegus coccinea* L. obtained from twigs collected outdoors. Mycelium developed in 11, and typical fructifications of *G. juniperi-virginianae* in 3. An unstated number of control calluses remained free of mycelium. Because successful culture of any rust fungus in the absence of the living host cells is of tremendous importance, confirmation of this report is highly desirable. Convincing evidence of this accomplishment would mark a triumph for tissue-culture techniques in plant pathology.

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DISCUSSION

Dr. Klein: Dr. Black has presented a completely logical evaluation of all the available facts, with an interpretation that is as valid as the one presented in our review. Certainly the answer is dependent on knowing more about the tumor-inducing principle and on the development of a better definition of virus than the ones now current. In numerous talks with virologists and bacteriologists, I have been struck not only by the absence of unanimity of definition but also by the fact that they, too, feel that present information is insufficient for the development of a precise definition of a virus. Yet, through the use of tissue culture a great deal of work can quickly be done to separate other replicable hereditary systems from virus. Of particular interest is the shadow world of cytoplasmic particles, mitochondria, microsomes, and plastids. All of these may be more easily examined *in vitro* than they have to date.

I wish that I could state, at this time, that the tumor-inducing principle is in that well-known bottle on my desk, but I feel that considerable time must elapse before any worker can make this statement. We have, however, made some progress in that direction. After over 3 years of work and 38 positive experiments, we are, at present, preparing a paper on the sterile induction of crown-gall tumors to supplement our initial report in 1953. In a recent short note, Dr. Pierre Manigault, of the Institut Pasteur, has confirmed this earlier work. Through our experiments, several additional characteristics of the tumor-inducing principle in crown gall have been worked out. It is, as postulated by Dr. Armin Braun, a large molecule that is non-dialyzable. It is not however labile at 32° C. as he reported, but its synthesis by the crown-gall bacteria is prevented at this temperature. Germane to this discussion is the report of Dr. Manigault that the tumor-inducing principle may be concentrated by high-speed centrifugation, a finding suggesting that its isolation is in the foreseeable future. Equally important is our finding that the tumor-inducing principle is insensitive to trypsin but is quickly inactivated by deoxyribonuclease. These are characteristics which would suggest that it is a "naked" DNA, which is unexpected in a typical virus. The tumors arising from appropriate treatments with sterile tumor-inducing principle and other tumor-inducing agents and factors involved in this transformation process have been carried as tissue cultures on standard unsupplemented medium for over 2 years. They are insensitive to, or inhibited by, auxins or coconut milk. Such insensitivity is one criterion frequently used to distinguish so-called normal tissue (properly, callus tissue) from tumor tissue. The grafting experiments with the carrot tissues, which we are using, are still incomplete because of certain technical difficulties, but presumptive positive results have been obtained.

The work on bacterial virulence, as reported by Dr. Deana Klein and myself in 1953, has been continued, and a second paper has just appeared in the *Journal of Bacteriology*. We have also received a personal communication that at least one other laboratory has confirmed these findings. In our recent studies we were able to run a series of quantitative transformation experiments for virulence in crown-gall bacteria. We have postulated that the ability of an individual bacterium to synthesize tumor-inducing principle (*i.e.*, to be virulent), is a multigene, multiallelic property in which host-range virulence and the degree of host-specific virulence are transformable characters.

Other studies have demonstrated, as we suggested earlier, that there is present in the juice of plant cells, a factor or factors necessary for the synthesis or, if Dr. Black is correct, the evocation of the tumor-inducing principle. This material is not host-specific, since beet juice and potato juice, etc., will serve as the source of this factor for the synthesis of a tumor-inducing principle effective for carrots.

It is hoped that before the next Decennial meeting Dr. Black's fundamental questions can be at least partially answered.

Dr. Nickell: Dr. Black has discussed at considerable length the desirability of being able to manipulate plant cells, as is possible with mammalian cells and microorganisms. The other day Dr. Boll discussed the desirability and the expectation that in the future we would be able to work with individual cells for the elucidation of certain nutritional problems and for the study of certain metabolic problems. Throughout this meeting you have heard reports on the observation of cells—single cells and groups of cells—as well as the discussion by Dr. Riker and others of the ability to take a single cell and make a tissue and we hope, eventually, a complete plant. For a long time the interest in our laboratory has been the reverse of the work done by Dr. Riker and his colleagues, that is, to take a tissue and make individual cells of it and continue to grow them. I would like to discuss the results which we have obtained. We started this work several years ago with many tissues. We have had most success with a tissue from pole bean. The initial aim was to grow plant tissues in liquid medium. For obvious reasons we wanted to increase their ability to grow, their growth rate. We wanted more absolute control of the material that we fed to them and of the materials that we reaped from them. We wanted to be able to study nutrition and metabolism; to obtain materials that are produced by plants. Some of these are of intrinsic value for research purposes, some are of very practical value because of certain types of metabolic products that are unique in plants. At the same time, we realized that the best way to do this would be to have the plants growing as discrete, individual cells. We started this by using submerged fermentation techniques and were pleased to find the cells started to come apart. These were *not* sloughed off cells incapable of further division, which we had to watch daily to see what they did because they were going to die. They continued to live. As time went on they continued as individual cells. We were able to transfer them serially and, with the passage of time, have been able to manipulate them just as one does a bacterial suspension. They have been discrete cells capable of being subcultured. At the present time our oldest clone is a little over 4½ years old. It has been subcultured continually through that time at 3- to 4-week intervals. This enables one to do growth-rate studies and metabolic studies. I hope it is a start in passing another hurdle in technique, a block that we have overcome, to enable us to do such things as Dr. Puck discussed so well in regard to mammalian cells at the last Growth Symposium.

I would like to mention very briefly the matter brought up by Dr. Black of crown-gall material which developed roots, differentiated, continued as roots, and then came back to an apparent crown-gall situation. The original culture was light beige in color and grew rather rapidly, and at one time several of the tubes spontaneously became completely filled with roots. At the same time it formed a type of tissue that was a very light green. After 14 months these roots, in some instances, again reverted to the original type of tissue.

Dr. Morel: One of the many points discussed this morning is whether the tumor-inducing principle in crown gall is a virus or not. First I would like to discuss the grafting experiments of de Ropp, since they are very suggestive of virus involvement. In this grafting experiment, the transmission of the tumorous character from a tumor cell to a normal cell was limited, so far as I know, to only a few layers of cells. de Ropp grafted a piece of tumor on normal tissue and noticed that the adjacent tissue became tumorous. But, so far as I know, in plant viruses anyway, when one transmits a virus to a plant, the virus never stays localized at one point but becomes systemic. I would like, since many virologists are here this morning, to discuss the question of transmission of a virus in a plant from one cell to another. I think that the main hypothesis is that the virus travels from one cell to another through the plasmodesmata. But I am not sure that in the crown gall there are any plasmodesmata. I made some experiments a few years ago to study the transmission of tobacco mosaic in crown-gall tumor tissue. I grafted a piece of crown-gall tissue inoculated with tobacco mosaic virus on a piece of crown-gall tissue of tobacco and studied the transmission of the virus. Even after several weeks I was not able to find the virus farther than a few millimeters below

the graft union. In that case it seems impossible for a virus to travel through the callus wall. But when this experiment is done on the entire plant, the whole plant becomes contaminated almost immediately. Perhaps if the tumor-inducing principle is a virus, its failure to become systemic may be due to the absence of these plasmodesmata. I would like to know if any cytologist has studied the plasmodesmata in crown-gall tissue.

I would like to comment on the very interesting experiment, reported by Dr. Nickell, on the transformation from a tumor callus to a root and then to callus again. Is the callus obtained from this root still tumorous? This could be easily checked by grafting experiments. Plant cells are known to recover from the crown-gall disease. This has been shown by Braun, with tobacco teratoma, and Dr. Riker reported, in this meeting, that he was able to raise an almost normal plant from a single cell from a crown gall of marigold. Perhaps in Dr. Nickell's experiment the root does not carry the disease any more and the callus obtained from it isn't tumorous. It is well known that normal roots are able to produce callus. This has been seen many times in our experiments with several species such as *Parthenocissus* and *Malva*. I would like therefore to know if the callus obtained from this root is still tumorous. And I think that grafting experiments like the fundamental experiments of White and Braun would be necessary to check that.

It is possible that some of the researches on the wound-tumor virus in tissue culture could shed light on the nature of crown gall. Dr. Black has suggested that in his cultures of sorrel the virus may no longer be present. It is very important that this hypothesis be tested. If the virus has disappeared and if the tissue is still tumorous, which can be easily demonstrated by the same grafting experiments, this will be comparable to the crown-gall disease. I myself tried, with Dr. Kassanis, to duplicate the one-step growth experiment with suspensions of cells using tobacco and tobacco mosaic necrosis virus, but as in the experiment reported by Dr. Black, I was not able to inoculate the virus into the isolated cells.

Dr. Brakke: I cannot add much to the story of the wound-tumor virus except to remark that this virus has been purified both from leafhoppers and from tumors of sweet clover growing in the greenhouse, and was found to be morphologically and serologically identical from the two sources. There has been no chemical work done on the purified virus.

I would like to make one comment based on Dr. Morel's remarks; that is, the lack of spread of virus after grafting is not limited to tissue culture. There are many plants in which viruses do not spread to the whole plant. This depends on many factors, such as growing conditions, type of virus, and point of inoculation.

Since I am primarily a virologist, the problems I come upon are mostly virus problems. I would like to mention a few that might perhaps be attacked by tissue-culture techniques. There are basic questions that need to be solved such as the relationship between the virus and the host cell. We have some other problems, for example, the stone-fruit viruses. There are about 50 of these that attack peaches, plums, cherries, and apricots. One of the main problems at present is that of identification. It is almost impossible to do any type of work from any viewpoint with these virus diseases unless you can identify them. At present it is not easy to do this. Some can be identified by symptoms or by their vectors. Others are identified by grafting diseased twigs onto certain "healthy" trees that are used as indicators. But the answer as to which virus is present depends on the healthy tree used as an indicator. This is quite an unsatisfactory method. If they could be purified, an attempt would be made to use particle morphology and serology for identification. The purification is difficult because there is no bioassay. Most of them cannot be transmitted mechanically. A few can be transmitted to herbaceous plants. With these, the use of morphology and serology of the infectious particles for identification might work. If tissue-culture techniques could provide an assay for these viruses and a source with a high concentration of virus and no tannin, it would certainly be useful.

In working on these trees another point has come up. Latent or symptomless viruses are widespread in many plants, which have been propagated by man vegetatively for a number of years. After talking to people working in this field, one wonders

if there are any plants that do not have viruses. Although the grafting of diseased twigs to healthy trees for indexing purposes, of course, is carried out with controls, it has been found that many "healthy" trees had viruses that produced no symptoms but would produce symptoms on susceptible trees. I might mention that there are some viruses transmitted through seeds, which may have latent strains and can be very widespread. The problems they present are similar to those of the latent viruses of vegetatively propagated plants.

In work with tissue cultures from such plants, it seems to me that the possible presence of these latent viruses must be kept in mind. The absence of noticeable symptoms does not mean a virus is absent. The widespread presence of these viruses and the symptoms they produce are sometimes quite untypical of what you would expect a virus symptom to be. This brings up the point of the definition of a virus. What are the differences between these viruses and the cytoplasmic particles? It may be hard to tell whether some of these particles really are a virus or a "normal" self-reduplicating cytoplasmic particle. These borderline particles are not limited to plants. Certainly the plant viruses that are transmitted by leafhoppers correspond to this type of particle as far as the leafhopper is concerned. There is a virus in *Drosophila* that induces only a CO_2 sensitivity. This is certainly an odd symptom, very specific, and one that you would not ordinarily find, and yet it is the only one produced by this virus. The symptoms produced by these viruses depend greatly on growing conditions. When such tissue is grown *in vitro*, the effect of the virus might be quite different and in some cases might be much more marked than in the intact host.

Dr. White: I should like to make one comment on this experiment of de Ropp, since it was done in my laboratory. The evidence of transmission of the agent from the bacterial crown-gall tissue to normal tissue is dependent on a single culture and was not duplicated by de Ropp or by anyone else, so far as I know. I think it is pretty shaky evidence. It depended upon the emergence of a bit of tissue, which appeared to be crown gall, from the normal tissue in the neighborhood of the graft and might therefore have resulted from growth of a separated cell of the inoculum and not from the normal tissue.

Dr. Black: May I comment on this experiment? Camus and Gautheret and McEwen have reported confirmation of de Ropp's experiment. There are two points in de Ropp's experiment which seem to me to be of particular interest. One of the points is that in one case, and in only one case, instead of the so-called induced (appositional) tumor appearing immediately adjacent to the scion tumor, it appeared at the end of the stem segment distal to the site of the graft. In this instance, it seems particularly unlikely that cells from the original tumor scion had actually penetrated through the stem to the opposite end. The other point of particular interest is the fact that de Ropp found that different clones of the sunflower crown-gall tissue in culture had different efficiencies in inducing these tumors. In particular, one line that had been derived from an induced tumor gave a much higher percentage of induced tumors than any of the others. This makes me wonder if some selection of virus mutants, more readily transmitted and more effective at induction, had occurred.

Dr. Woolley: One of Dr. Klein's statements brought the following question to mind. He pictured this agent as being naked deoxyribonucleic acid. I wonder whether any attempts have been made to put an overcoat on this DNA and get an infectious particle.

Dr. Klein: I should have put quotes around the word "naked." What I actually meant was that, in many of the bacteriophages, the phage is insusceptible to the action of deoxyribonuclease (DNase) until it is acted upon by something like trypsin to hydrolyze the "overcoat." The fact that, in our studies, trypsin was completely without effect would indicate that DNA was immediately available to the activity of the hydrolytic enzyme. No attempts have been made to put protein "overcoats" on the tumor-inducing principle, primarily because we haven't enough of the material with which to do any work, but obviously this is one of the things that can and will be done.

Here is what we did and I think that it may partially answer the question. After we had collected the culture supernatant containing the tumor-inducing principle, it was treated with trypsin, dialyzed to remove amino acids that might have been freed by

hydrolysis, and then sterilized by filtration. The proteinase was still present throughout the whole experiment. This would suggest it is unlikely that there is a relationship between protein and TIP because the amount of protein remaining is negligible.

Dr. Ball: Is there any relationship between the infections of the virus and the well-known mechanism in the bacteriophage where the inciting virus more or less takes over the protein metabolism of the host cell? I should like some discussion of that in the answer. With regard to Dr. Morel's point about plasmodesmata, so far as I know from my work in anatomy, there is only one instance of living plant tissues that are supposed to be without these protoplasmic connections, that is, certain young cork cells before they lose their protoplasm. All other cells, according to older work, have well-developed protoplasmic connections between the cytoplasts of the cells.

Miss Owens: Would any of the members of the panel wish to comment on reports in the literature concerning migration of mitochondria from one cell to another?

Dr. Klein: I would be delighted to discuss this point because it is what I have been postulating. A number of years ago, actually in the 1940's, there were several studies which indicated that, concomitant with the processes leading to the formation of crown-gall tumor cells, there were alterations in the number and the morphology of the mitochondrial particles found in the cells. These studies were not carried much further, except to indicate that these altered numbers and altered morphological forms of the mitochondria were maintained throughout the life of the tumor. These studies were primarily done on plant cells *in vivo*, *i.e.*, stems of tomato, Jimson weed, and a few other plants. No study has been made to date to determine whether, in the change from normal to tumor, as examined with tissue culture, there is maintenance of the state of altered mitochondria, nor in the studies of transmission by grafting has this type of work been done. We are at present attempting, in our laboratory, to isolate the mitochondrial and microsomal fractions from normal, habituated, and crown-gall tissue of *Parthenocissus*, by differential centrifugation. Not only do we plan to do some studies on the phosphorus metabolism of the particles, as measured by P:O ratios, etc., but we are very interested in attempting to put these fractions into healthy cells and see what happens. Some studies have been done by animal workers. Stasney, Cantarow, and Paschkis in 1948–50 took a fraction from rat tumors and claimed that they were able to transmit the tumor into a healthy rat, a result that was questioned by Dr. George Klein. Perhaps Dr. Klein could indicate the status of this attempt to put mitochondria into the animal tissues, which would give us a lead as to whether mitochondria or similar fractions might actually be the continuing cause of crown gall. For example, if we assume that the tumor-inducing principle is solely an inducer, rather than being both the inducer and the continuing cause (an assumption for which I think we have some good evidence), it would suggest that, as an inducer, it acts to alter some cytoplasmic and heritable character or to evoke from the plant cell a latent virus or latent hereditary particle, this particle being the "cause" that maintains the cell as a tumor cell.

Dr. Skoog: If I followed you correctly, this tumor-inducing principle not only has remarkable biological properties but also remarkable physical properties. Did you say that it is stable to heat (30° C.) but is inactivated by cold?

Dr. Klein: The physical properties of the tumor-inducing principle are as follows: It is stable to about 65° C. for 10 minutes, unstable at 80° C. for about 2 minutes, and slowly broken down at refrigerator temperatures, over a period of 24 hours. It has not been purified.

Dr. Black: I would like to comment briefly on Dr. Ball's question. There is no doubt that plant viruses exert a tremendous effect on the host plant's protein metabolism, although I must confess that I don't understand the statement, sometimes made, that the introduced virus uses the metabolic equipment of the host cells, without attributing any metabolic ability to the virus itself. It seems to me that the virus, when it is introduced, brings about so many changes that we might well suspect that it controls a number of its own independent enzyme systems. I think there is beginning to be evidence indicating that viruses do cause to appear in cells, enzyme systems that weren't there before. Indeed, it would be very remarkable if the

changes induced by the virus could be brought about without the virus introducing factors that control independent enzyme systems and that were not there previously. In the case of tobacco mosaic virus, I recall that under certain conditions about 80 percent of the protein in extracts from diseased plants is virus protein. This illustrates the extent to which virus may interfere with the protein metabolism of an infected plant.

I would also like to comment on the question regarding the transmission of mitochondria in plants. A number of years ago, Woods and duBuy published papers in which they interpreted their experimental results as indicating a more or less continuous spectrum between viruses and mitochondria in plants. I think that it is not possible to demonstrate that there is such a continuous spectrum by the techniques they used. It is always possible to interpret their results as follows: Where they got transmission, they actually were working with an unrecognized virus, and where they did not get transmission, they were dealing with a variegation that was, in fact, the result of mitochondrial or plastid abnormalities of some sort. I don't think it is possible, by grafting experiments, to demonstrate a continuous spectrum between the two extremes.

Dr. Bang: In regard to Dr. Black's suggestion that this agent might be carried by the bacteria, I would like to ask for some discussion of the question as to whether this could be carried by means of lysogeny. Since Dr. Lwoff is here, I should like to hear particularly whether there might be any specific experiment performed that might be pertinent to the crown-gall bacterium?

Dr. Lwoff: Dr. Black has discussed the problem of the definition of viruses. It is a very important problem, because the definition we adopt might influence the way of attacking the problem experimentally. I have tried to find out what the essential difference is between a virus and a nonvirus. My conclusion is the following: The virus particle is a nucleoproteinic particle with only one nucleic acid, and it is reproduced from its nucleic acid. This definition allows one to separate viruses from classical organisms. In all classical organisms two types of nucleic acid are present, DNA and RNA. In all classical viruses only one type is present. All organisms are reproduced from the totality of their constituents, while phage, and probably all viruses, are reproduced from their nucleic acids. Now this definition is, of course, not sufficient, because when we think of virus, we think of something that produces a disease. We have to add pathogenicity to the definition of a virus. But there are some difficulties to this addition because, in lysogenic bacteria, the genetic material of the phage behaves exactly as if it were a gene. It is located on a specific spot of the bacterial chromosome. One might discuss whether or not the pro-phage, which is the genetic material, is or is not a virus. It is not a virus in the sense that it is not infectious and that it doesn't produce a disease of the bacterium, but does modify the bacterium. When we look at the problem of the possibility of viruses being involved in the production of tumors, we must remember lysogeny. A tumor cell is a cell that is modified, just as a lysogenic bacterium is a bacterium that is modified, by the presence of a foreign genetic material. The most important thing to know is at what level we consider the disease to be. The malignant cell *in vitro* may be different from a normal cell but it is able to reproduce and, for all practical purposes, it is healthy. In a virus-induced malignancy, the virus modifies the cell. The malignant cell, considered by itself, is not ill. It is an altered cell which has lost its ability to be controlled by the factors of correlation. It is the cell as modified by the virus (or pro-virus), which is pathogenic for the organism. Here, as in the case of lysogenic bacteria, the virus (or pro-virus) is not pathogenic but is a "modifier." When we consider these data, it becomes more and more difficult to define a virus, and perhaps the best definition is that a virus is something that does something. When we try to define a virus, the properties of the 3 phases of the life cycle, infectious, vegetative, and pro-viral, have to be taken into account.

Dr. Syverton: I should like to ask Dr. Klein whether he has attempted to learn the nature of the agent, or modified material, that resulted on transfer to new plants in different or altered manifestations? I ask this question because of experiences with

myxoma virus. Myxoma virus can be modified by physical agents; for example, heating to a temperature of 80° C. yields a modified virus that provokes a fibromalike disease. Myxoma virus, on the other hand, if kept at refrigerator temperature, loses its infectiousness.

Dr. Klein: These experiments are the ones that, as I mentioned, were incomplete because of technical difficulties. We do have the sterile tumors arising from the treatment of this isolated tumor-inducing principle, and to complete the proof that they are actually tumorous we must show that they are capable, when grafted, of proliferating as tumors without fusing with the host as does typical callus tissue. The problem of using carrot has been, of course, that it is extremely difficult to graft a piece of carrot tumor-tissue culture onto a piece of carrot root. The stem in the edible carrot is just the top part of the root and is not enough to work with. I have just received some seed of another species of *Daucus* that has a good stem. These experiments will be done this winter, and I will be very interested in what type of tumors develop.

Dr. Henderson: I would like to report some results which might have implications for Dr. Klein's tumor-inducing principle, something we would like to call *tumor-progression principle.* The sunflower tumor tissues, when accidentally exposed to 32 or 33° C. over a period of about 6 weeks (when the air-conditioning system didn't work in this particular room), began to grow very poorly during the next transfer period. We almost lost the tissues completely. So we put these tumor tissues on Gautheret's medium and a supplement, including vitamins, amino acids, and indoleacetic acid. We put supplement in the medium of certain tissues that were dying out and we omitted it from the others. It turns out that those tissues, which we formally call crown-gall tumor tissues and to which we added supplement, immediately began to come back, and grew normally at the usual rate in the next transfer period. Those to which we did not add supplement continued to go down in weight until they reached a point of mere maintenance with no further growth. I am only suggesting the possibility that the tumor-inducing principle was destroyed at this relatively high temperature and that it could now be replaced in some way by adding back to the tissue some of these nutrients, either the vitamin mixture or the adenine sulfate, which was the only nucleic acid principle, or the casein hydrolysate. The point we are trying to make is that there is something between the tumor-inducing principle from the bacterium and the tumor-progression principle that is carried along from transfer to transfer in the tumor tissue. While we have this single-cell culture of Riker, Steward, and others, it seems to me that this is the time to begin to experiment with the single cells and the bacterium or extracts from the bacterium to see if we can get a valid tumor culture from a single cell. Then we will not have to guess whether we have some normal cells still carried over into the tumor cultures and, therefore, whether we are getting normal cells and normal organs from tumor tissue.

Dr. Gautheret: I want to point out some questions concerning the differentiation of organs in crown-gall tissue cultures. In his first work, Braun thought that he was observing a recovery of the crown-gall cells. But Nickell and Démétriadès observed that the roots, which are differentiated by crown-gall tissue, can subsequently form callus cultures similar to crown gall. This seems to demonstrate that there is no real recovery. This is not quite certain because it would be necessary to test all the physiological and pathological properties of these tissues after passage through the root or through the stem. I want to know if this test has been made, that is, if one has compared the properties of the first stage and the properties of the second stage concerning, for example, the growth substance requirements and also the properties that this tissue shows upon grafting on a healthy plant. This is probably the same as the tumorous tissue induced by bacteria-free crown-gall tissue in de Ropp's experiments. It is necessary to know if these tumors induced by bacteria-free substances have the same physiological properties as the true crown-gall tissues.

Dr. Nickell: Since it has been asked, twice, I can say that the material which arises, after the crown-gall tissue has differentiated into roots, and has then reverted to a tumorous tissue, is physiologically the same as crown gall. The grafting experiments have not been done.

The Response of Mammalian Cells in Culture to Virus Infection [1,2,3]

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This review of the responses of cultured mammalian cells to virus infection is intended to provide background for discussion of the morphological and functional changes that occur in cells infected with viruses or other microbiological agents. The discussants, Doctors Dixon, Bang, Lasfargues, and Weller, are eminently qualified to broaden and extend the subject.

For the virologist, the infected cell and its intracellular virus is the prime focus of interest. We define viruses as nucleoprotein particles, variously shaped, ranging in size from 8 to 300 mμ, and capable of parasitically inducing their own replication by living susceptible cells. Like their hosts, viruses possess distinctive genic components which ensure replication yet permit mutability. These replicating particles of extrinsic nucleoprotein alter cellular metabolism and morphology to result in disease of the cell or organism. The *in vitro* cytopathogenic effect of virus is used primarily by virologists as a practical indicator of virus activity. Cell culture, however, has an even greater potentiality for analytical consideration of the host-parasite relation between virus and cell, because chemical and physical factors are subject to purposeful manipulation. Cell culturists have long been concerned with the nutritional and environmental factors that influence cellular metabolism and morphogenesis; virologists also must comprehend these factors to interpret adequately the response of cells to infection. It is fair to say that the demands of the virologist have accelerated the development of a science of cultured cell biology out of the earlier use of *in vitro* culture as an extension of animal anatomy and physiology. Theoretical studies of the cell-virus relationship initially were delayed until methodological advances could provide the cells and cultural conditions peculiar to the virologist's needs. Precise studies of cellular nutrition laid the foundation for definition of cellular response to virus infection in terms of cellular metabolism. The recent reports of Eaton *et al.* (*1*, *2*) on the influence of thyroxin and osmotic pressure on *in vitro* growth of influenza virus,

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of Kovacs (*3*) on the effect of poliomyelitis virus infection on cell enzymes, of Eagle and Habel (*4*) on the nutritional requirements for propagation of poliomyelitis virus in HeLa cells, and of Gifford and his associates (*5*, *6*) on the use of manometric method for studies of chemical inhibition of virus synthesis, all reflect growing interest in biochemical evaluation of cellular response.

It seems a long time since the Conference in 1946 when the 4 techniques for the study of virus-host-cell relations were: *a*) The Harrison coverslip–hanging-drop technique (*7*), suitable for short-term experiments; *b*) the Burrows-Carrel flask–plasma-clot technique (*8*) that provided nutritional and environmental conditions conducive to continued propagation and maintenance of cells; *c*) the Gey roller-tube–plasma-clot technique (*9*) that permitted use of large numbers of cultures and maintenance of cells by successive serial transfer in a relatively constant environment; and *d*) the Maitland technique (*10*) that substituted suspended tissue fragments for explants. The Maitland technique was widely applied, usually by virologists without previous experience in cell culture. Many viruses were propagated, but because some could not be propagated in suspended fragments of the host tissue as indicated by the *in vivo* trophism of the virus, the impression was received that they could not be propagated *in vitro*. The fallacy of this impression was demonstrated in 1949 by Enders and his associates (*11*) when they cultivated poliovirus in human-tissue cultures of non-nervous origin. This finding encouraged virologists to examine the potentialities of the cultivated cell as a test host; the end result, as you know, has been the incorporation of cell-culture technique into virology as a primary and indispensable tool. The mass-produced cell culture required by virologists was made possible through: *a*) antibiotics to control contamination, *b*) trypsin to disperse cells, with the method originated by Rous and Jones in 1916 (*12*) and reintroduced by Scherer, Syverton, and Gey in 1953 (*13*), and *c*) cell-dispensing apparatus simplified from that devised by Evans, Earle, and associates (*14*) to provide replicate cultures. As a result, primary cell cultures of human or animal tissue and a variety of cells in continuous culture have largely replaced animals and embryonated eggs for isolation of viruses, assay of antibodies, analysis of the physiology of virus infection, and investigations of the bases of antiviral antibiosis.

To the participants and guests of this Conference, undoubtedly the protagonist of great interest in the host-cell-virus relationship is the cell. Strains of stable and pure-line cells in continuous culture largely and properly have replaced the primary cell or tissue culture for examination of virus-cell interactions. For example, 9 stable human-cell strains are maintained in our laboratory to provide 4,000 cultures weekly for investigative studies. The human strains include 1 of esophageal epithelium, 2 of normal-fibroblast origin, 2 derived from carcinomas of the cervix and lung, 2 of liver epithelium, and Chang's conjunctival cell strains. Several other strains under development descend from swine, mouse, ferret, and rabbit origin. Modification (*15*) of Puck's method

for single-cell isolation (*16*) has provided 5 of the 9 stable human strains in multiple pure lines (clonal lines). At least 10 other stable human strains are available in other laboratories (*17–25*). It is timely to remind you that the simple procedure for dispersion of mammalian cells by trypsin led to use of *a*) cells as monolayers in bottles for production of cells and viruses, *b*) cells in tubes for bioassay, *c*) single cells for harvest of virus clones and plaque-purified virus strains, and *d*) single cells for derivation of clonal or pure-line cell strains.

Because virus multiplies only inside an appropriate host cell and transforms that cell and its complex set of enzymes into a factory for replication of a foreign nucleoprotein—the virus—it is not surprising that viral replication is accompanied by morphologic evidence of cellular injury. Contrariwise, it is surprising that the range of injury commonly referred to as a cytopathogenic effect or a cytopathic change should be so great. The spectrum of cellular changes from virus infection, manifest grossly or microscopically, extends from simple alteration in physiologic activity to total necrosis; from necrosis to hyperplasia; and from hyperplasia to neoplasia. Cell culture reveals the intimate nature of virus-induced damage. Currently, cytopathic alterations of cultivated cells are recognizable as modal effects at 5 levels, which will now be described.

The most severe effect, *total destruction*, proceeds from cellular rounding and withdrawal of processes to nuclear shrinkage and fragmentation of chromatin to final cellular disruption in periods ranging from several hours to a few days. Complete destruction is the usual result of infection by viruses of the poliovirus and Coxsackie B groups, Coxsackie immunotypes A-9, 11, 13, 15, and 18 viruses, and some of the ECHO group of viruses. This infectious necrobiosis can affect both epithelial and fibroblastic human cells.

The next lesser type of injury is seen in cultures showing some cells necrotic and others shrunken with pyknotic nuclei and altered staining properties. This *subtotal cellular degeneration* is produced by viruses of the arthropod-borne encephalitogenic group and by Newcastle disease virus. St. Louis and Japanese B encephalitis viruses, on the other hand, can infect some cell strains without obvious cytopathic effect (*26*).

Viruses of the herpes-B-pseudorabies group slowly produce a *focal degeneration*, with centrifugally progressive development of intranuclear inclusion bodies. Evolutionary stages of inclusion-body formation can be seen in cells variously distant from the periphery of each plaque of destruction. Occasionally, one sees amitotic division of nuclei. Vaccinia virus is similar in action except that inclusions occur in the cytoplasm, and nuclear changes do not precede cellular necrobiosis.

Epithelial or fibroblastic cells infected by adenoviruses exhibit *cytopathic changes without cessation of cellular metabolism*. Infected cells accumulate fluid, distend, and aggregate into refractile clumps; thereafter the clumped cells become increasingly granular and degenerate. Finally, the clumps peel off the glass and float in the fluid. Epithelial cell cultures completely succumb in 2 to 7 days after the first evidence of damage appears; fibro-

blastic cells may respond more slowly over a period of several weeks. The injurious process is unique in that metabolic activity of infected cells, despite their degeneration, continues to lower the *p*H of the culture medium.

The changes known as *foamy degeneration* have been described by Rustigian (*27*), Enders and Peebles (*28*), and others, as induced by measles virus and simian orphan viruses. Affected cells coalesce ultimately into syncytia sometimes including 40 or more nuclei. The damage accumulates slowly and spreads outward from isolated foci; cell boundaries disappear; nuclei clump, and cytoplasm becomes confluent and vacuolated with torn and foamy appearance.

What processes of intracellular disease are reflected by these visible symptoms is not known. The exemplary efforts of Ackermann (*29*), Lwoff (*30*), Barski (*31*), Harding (*32*), Reissig (*33*), Dunnebacke (*34*), and their associates, to characterize and analyze the destructive effects of poliovirus emphasize the problems involved in the study of cellular pathogenesis.

The rapidly expanding field of cellular virology has applied as well as fundamental scientific value. Practical applications of cell culture are helpful to the physician and to the epidemiologist (and indirectly, to the average citizen) for isolation of viruses and accurate assessment of individual or group immunity. The specific diagnosis of poliomyelitis, for example, and of many other virus infections is accomplished readily by cell-cultural methods. Virus is isolated and recognized by its ability to affect and destroy susceptible cells in culture, while the capacity of homotypic antibody to neutralize cytopathogenic potential of the virus permits identification of isolates. Cell cultures bring diagnostic virology within reach of all laboratories and offer reliable results obtainable almost as quickly as those required for diagnosis of other microbial infections.

Increasing use of cell cultures, while solving old problems, has introduced new ones. Viruses other than poliovirus that produce cytologic changes have been isolated in all laboratories with cell cultures for virus diagnosis. These isolations have in a few instances turned up the familiar viruses of herpes simplex, influenza A and B, mumps, measles, or varicella, but they have yielded new cytopathogenic viruses. These cytopathogenic agents, which have all been isolated from the alimentary tract (throat or intestine), include at least 4 groups: *a*) *C viruses* (Coxsackie group) produce paralysis and death of mice less than 24 hours old. Twenty-four antigenically distinct types of C virus in 2 subgroups, A and B, are known; 6 immunotypes of subgroup B and immunotypes 9, 11, 13, 15, and 18 of subgroup A are cytopathogenic. The viruses of subgroup B allegedly cause epidemic pleurodynia and aseptic meningitis, while those of subgroup A are responsible for herpangina and possibly other human illnesses. *b*) *Adenoviruses*, a newly discovered group of 12, produce some forms of respiratory disease and keratoconjunctivitis. These viruses are known alternately as the ARD (acute respiratory disease) or APC (adenoidal-

pharyngeal-conjunctival) group. Adenoviruses share a complement-fixing antigen but are separable by neutralization tests. *c*) The *enteric cytopathogenic human orphan* (*ECHO*) *group* includes 14 antigenically distinct viruses. The properties shared by these newly discovered viruses are summarized by the Committee on the ECHO Viruses of The National Foundation for Infantile Paralysis, Inc., (*35*) as follows:

> "(i) They are cytopathogenic for monkey and human cells in culture. . . . (ii) They are not neutralized by pools of the three types of poliomyelitis antiserum. (iii) They are not neutralized by antiserums for Coxsackie viruses that are known to be cytopathogenic in tissue culture, and they fail to induce disease in infant mice . . . . (iv) They are not related to other groups of viruses recoverable from the alimentary tract (throat or intestine) by inoculation of primate tissue culture, such as herpes simplex, influenza, mumps, measles, varicella, and the ARD . . . or APC . . . group. (v) They are neutralized by human gamma globulin and by individual human serums; this indicates that they infect human beings."

d) Laboratories that use monkey kidney cells for diagnostic purposes or for the preparation of polio vaccine must consider an additional group of immunologically distinct cytopathogenic viruses indigenous to monkeys. These problem viruses, isolated from tissues or alimentary tract by Rustigian (*27*) and others, are known from Hull, Minner, and Smith's studies as the S.V. or *simian group* (*36*).

New viruses, which Duran-Reynals called collectively "orphan" viruses because they were viruses in search of disease, ultimately may concern the practicing physician as well as the laboratory worker. A number of laboratories, under sponsorship of The National Foundation for Infantile Paralysis, Inc., and the United States Public Health Service, share responsibility for the designation and separation of the ECHO group and other groups of orphan viruses and for establishing each newly discovered virus as the causative agent of a specific human disease.

Finally, in summary, it is evident, even from this brief consideration, that ability to cultivate mammalian cells *in vitro* has greatly expanded our knowledge of cellular response to virus infection. It is anticipated that improved methods for detection of subtle differences in cellular vitality and virus synthesis will be forthcoming, and that continued study of viral infection at the cellular level will yield the antimetabolites or antibiotics so long desired for treatment of virus infections of man and animals.

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DISCUSSION

Dr. Dunnebacke Dixon: I would like to suggest that the cell itself may affect or control the manner of the cytopathic expression of virus infection and to discuss some work we have been doing at Berkeley. We have compared, under similar experimental conditions, the cytopathic changes in a variety of cells after inoculation with poliovirus types I, II, and III. We attempted *a*) to characterize the sequence of cytopathic changes in the cells, *b*) to compare the effects of the 3 types of poliovirus on the same and on various cell types, and *c*) to determine the time and stage in the sequence of cytopathic change at which the newly formed virus is released into the medium. We found that the 3 types of poliovirus appear to affect cells in a similar manner (*1*) and that the cytopathic changes are similar to those described by Barski *et al.* (*2*), Harding *et al.* (*3*), Beale *et al.* (*4*), and Reissig *et al.* (*5*). A typical experiment using HeLa cells is represented in figures 1, 2, 3, 4, plate 59. A control culture is shown in figure 1. The infected cells were classified in 3 main stages. In the first (fig. 2), the nuclear membrane appeared wrinkled and there was no obvious shrinkage of the cytoplasm. In the second (fig. 3), the cytoplasm was partly constricted and the nuclear staining material was condensed. In the third (fig. 4), the cell was rounded and the formation of a new structure, which has been referred to as a "lesion" and "inclusion" or a "cytoplasmic mass," was apparent. This new structure was centrically located and appeared to have displaced the condensed nuclear staining material. These changes were observed in inoculated HeLa cells, monkey kidney cells, and 8 strains of human fetal cells. When we compared the percentage of cells in each of the cytopathic stages with the percentage of the total yield of virus released into the medium at various time intervals, we found that the cells probably had undergone the 3 major cytopathic changes before the virus was released. Our data indicated that the sequence of cytopathic changes and the pattern of virus release were the same for the 3 types of poliovirus and for the various cell types used. However, when we studied the process of polio infection in human amnion cells, we found that they undergo a completely different series of cytopathic changes (*6*). In the control cultures (fig. 5, plate 59) human amnion cells were spread out thinly on the glass and had discrete nuclei and nucleoli. In the infected cultures, no gross change was obvious in the first 4 hours. By 6 to 8 hours (fig. 6, plate 59) the nucleoli had become indistinguishable in the majority of cells. By 12 hours (fig. 7, plate 59) the cells in which the nucleoli had become indistinguishable had begun to withdraw their cytoplasmic processes. In 12 to 19 hours (fig. 8, plate 59) from 10 to 15 percent of the cells appeared to have nodules, projections, or bubbles on the free surface. It seems that these projections appear as blebs on the surface, and then become nodular and attached by stalks as if they were being released from the cell. When we compared the percentage of cells in each cytopathic stage with the percentage of virus released at various time intervals, we found a very close correlation between the appearance of cells with nodular projections and the release of newly formed virus into the medium. We interpret these data to mean that the amnion cells contain some quality or factor that is different from the other cells studied and can influence the structural cytopathic expression of poliovirus infection.

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PLATE 59

FIGURE 1.—HeLa cells: control culture.

FIGURE 2.—HeLa cells, 4 to 6 hours after inoculation. *Note* wrinkled appearance of nuclear membrane.

FIGURE 3.—HeLa cells, 6 to 8 hours after inoculation. The nuclear staining material is congregated to one side of the cell and the cytoplasm is partially constricted.

FIGURE 4.—HeLa cell, 8 to 10 hours after inoculation. The nuclear staining material is condensed into a small mass, centrically located and adjacent to the newly formed "lesion" which appears lighter staining.

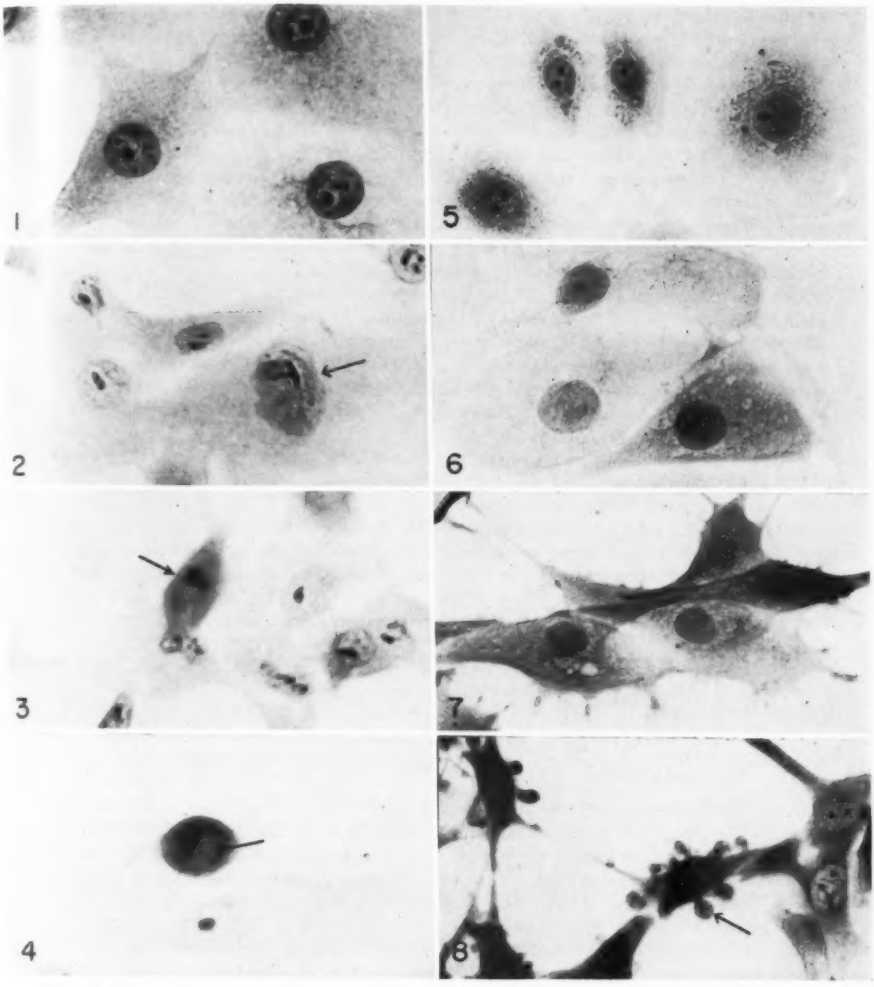
FIGURE 5.—Human amnion cells, control culture.

FIGURE 6.—Human amnion cells 6 to 8 hours after inoculation. The nucleoli are indistinguishable.

FIGURE 7.—Human amnion cells 10 to 12 hours after infection. The cytoplasm is partially constricted.

FIGURE 8.—Human amnion cells 12 to 19 hours after inoculation. Nodular projections appear on the surface of the cells.

Photographs are taken from Dunnebacke (*1, 6*).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Dr. Bang: The response of mammalian and, we may add, avian cells to viruses may be thought of in terms of the reaction of 1 cell to an invading agent or of the reaction of a group of cells to a common infection. The group response would obtain in colonial cultures of cell lines in roller tubes as well as in cultures of organized tissues. We believe that the present body of tissue-culture knowledge can be used for the study of the specificity of virus infections for given tissues or organs or cells. We will, however, try to discuss the tissue and organ aspects later and to concentrate at present on the pathogenesis of infection within an individual cell.

We had thought that the available data on the cell as host could be arranged to show the action and reaction of a cell's component parts from periphery to nucleus, but the lack of information on many crucial points prevented this. We will begin therefore with the areas of ignorance and work toward a summary of what is known so far.

At the periphery, viruses are absorbed onto cells (*1*) and are presumably engulfed in them. The process has not been seen. Cells have been seen to engulf particulate matter and fluid and, in the process of pinocytosis (*2, 3, 4*) or fluid-swallowing, we may presume that active virus material is taken into the cell. The electron microscope has been used to observe pinocytosis of the virus of Newcastle disease (*5*). The significance of the amount of virus involved in this event is not known.

Again at the periphery, cells in culture may have undulating surfaces and may in addition have a variety of processes, from short and stubby to very long and thin. Virus activity may destroy these processes or the cell may respond to virus by greatly modifying its normal processes or microvilli. Chick epithelial cells, for example, respond to influenza and Newcastle virus by a remarkable extension of microvilli into filaments so long and laden with virus that we cannot say how much of a filament is host cell and how much is virus (*6, 7*).

Peripheral destruction of the cell may, of course, be seen in the phase microscope. When encephalitis or poliomyelitis virus destroys the cell periphery, material continues to be carried inward to the paranuclear area or Golgi zone. If cell destruction has not proceeded rapidly with this inward invasion, the paranuclear area may become "hypertrophied" in the form of large inclusions (*8, 9*). Such inclusions, in the same part of the cell, have been seen in other virus infections such as the Rous sarcoma (*10*) (fig. 1, plate 60) and cells degenerating from disease or age (*11*). They also have been seen in the spontaneous tumor cell T–333 (Dr. Gey's), which nevertheless grows very nicely (fig. 2, plate 60).

Incidental to the penetration from the periphery inward (figs. 3, 4, plate 60), we know that a virus may produce peripheral destruction alone in 1 cell and yet may completely take over the cytoplasm of another. Once established in the cytoplasm by one means or another, the virus may still be isolated from the main stream of the cell processes by being walled off into a vacuole by the cell. This occurs with the psittacoid group.

Living mitochondria are now commonly studied in motion pictures, and several of of us have seen particles within or associated with mitochondria (*13, 14*) (Dr. Bernhard's studies on chicken-tumor virus is the most recent as well as the best). But we do not know yet whether any of the familiar or exotic viruses grow in mitochondria or parasitize or selectively destroy them. Considering the current biochemical interest in them, this is a startling area of ignorance.

Advisedly omitting the endoplasmic reticulum, we come to the nucleus. At this point I shall discuss the membrane and its double nature, which everyone acknowledges but which everyone does not interpret as the same kind of "double." If there are 2 membranes, 1 of which is cytoplasmic and 1 nuclear, then the fluid interspace between the 2 membranes would vary as the nucleus slipped around in the living plastic cell. Virus that spills from either the cytoplasm or the nucleus into the interspace would accumulate there before penetrating the next membrane. We believe that the pictures published by Dr. Reissig and Dr. Melnick (*15*) on herpes-B virus may be interpreted in this context. In our own studies of pathological cells, we have seen large paranuclear blebs in which the separateness of these 2 membranes is apparent (*13, 16*). Dr. Weller will cover much of the morphological processes within the nucleus, and we will therefore not attempt a discussion here.

Groups of cells that could reasonably be expected to be uniform biologically do not necessarily react to virus in a uniform way. One of the current puzzles in this area is the story of experimentally reproducible chronic infection. Dr. Gey and I found some years ago, that with some strains of cultured rat cells not all cells died of infection and those that survived sustained a chronic infection potentially *ad infinitum* (*17*). The course of this particular infection is conditioned by the presence or absence of embryo extract in the culture medium. Long-term experiments on this chronic infection are summarized in table 1.

TABLE 1.—*Late results of temperature and embryo-extract variation on infection of rat fibroblasts (Ansat 72) with Eastern equine encephalomyelitis virus*

Expt. no.	Effect on cells at following incubation temperatures: 31°		37°		41°	
	10 Percent embryo extract	No embryo extract	10 Percent embryo extract	No embryo extract	10 Percent embryo extract	No embryo extract
15	Poor recovery	Dead	Recovered	Poor recovery		
17	Chronic infection	Dead	Recovered	Dead		
25		Dead		Recovered		
28	Chronic infection		Moderate recovery			
32		Dead		Recovered		Dead
33		Dead		Recovered		¾ Cultures dead

In conclusion I would like to emphasize that a given virus may have different reactions with different cells, and that strains of a virus may vary in their virulence for the same host cell so markedly that they produce very different end results. A corollary precaution is to avoid the notion of 1 type of virus lesion. Our task in analyzing the pathogenesis of virus infections is, rather, to understand the relation between the intimate constitution of the living reacting cell and the variety of effects that parasitic agents have upon it.

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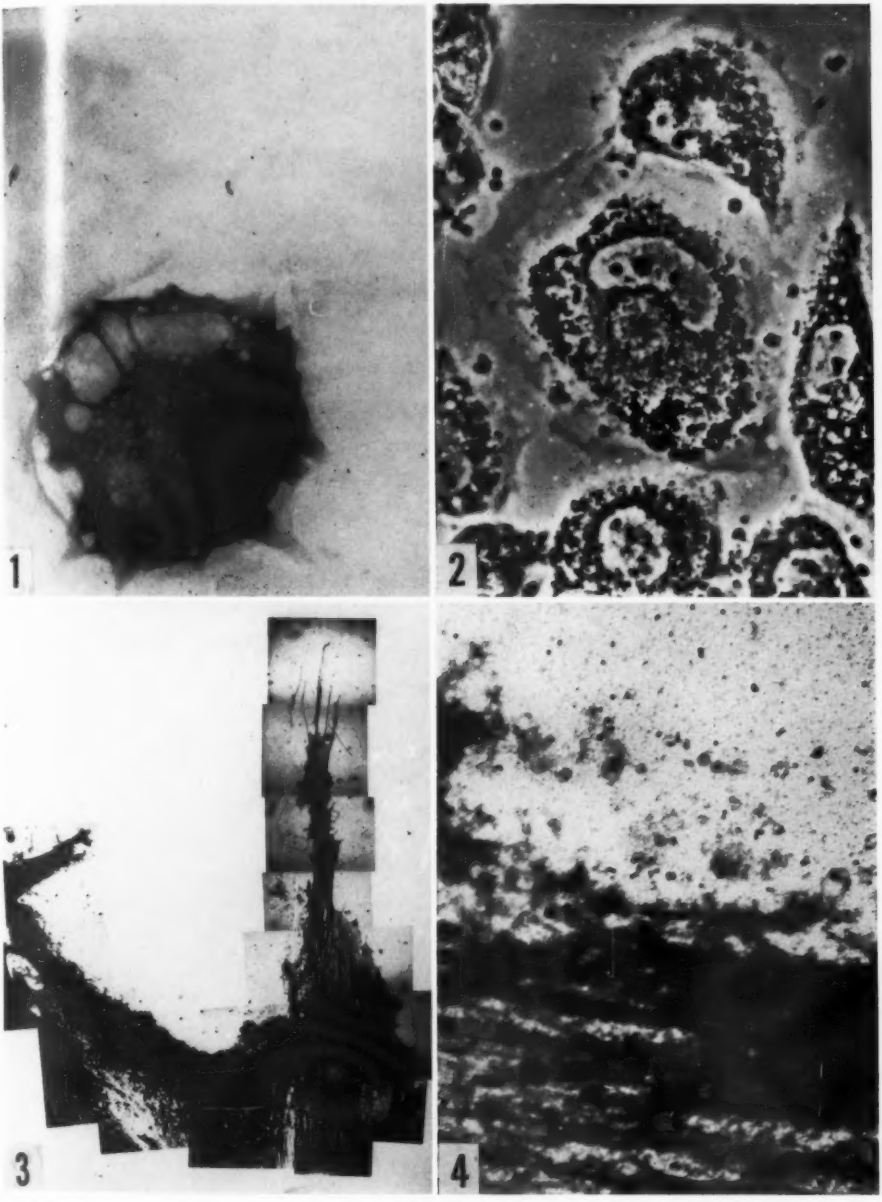
Plate 60

Figure 1.—Chicken fibroblast infected with Rous sarcoma virus, stained with Giemsa. × 500

Figure 2.—Rat tumor cell T-333, phase micrograph of living cell. × 500

Figure 3.—Composite electron micrograph showing destruction of rat-tumor cell. × 1,000

Figure 4.—Enlarged area from same. Virus particles seen in the area of destruction. × 14,500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Dr. Lasfargues: The clear and precise review presented by Dr. Syverton shows how much virology has been dependent on the development of tissue-culture techniques. In the past decade, virologists in search of a suitable medium to propagate these pathological agents have been fortunate enough to witnses the development of stable cellular strains that can be easily handled with standardized methods. The recent work of Puck, introducing a new technique of obtaining clonal growth of mammalian cells, will probably increase the stock of strains already available. Thus, one of the difficult problems of virus cultivation is practically solved. Other problems, however, are still challenging the research worker. Dr. Syverton emphasized, as well as Dr. Bang, the cytopathogenic effects of the viral agents and described 5 different levels at which they can be recognized. Most of the actual methods of diagnosis and titration are based on the observation of such morphological changes. However, when the modifications of cellular morphology are so subtle that they result in a slight alteration only, one is obliged to rely on the usual techniques of immunology. If those methods fail, which has often been the case, we are confronted with the problem of simply detecting the virus in the culture. We have been facing this situation in our attempts to multiply the distemper virus and at present we are more acutely concerned with the agent of the mouse mammary carcinoma. Although the identification of this agent with a true virus is still an open question, we will retain this denomination, supported as it is by the agent's filtrability, by electron microscopic preparations that have shown repeatedly the presence of small spherical particles of about 100 mμ in diameter, and by the physical properties that are consistent with the virus hypothesis. Our success in growing important quantities of normal mouse mammary epithelium rapidly convinced us that there is no major morphological difference from neoplastic cells at the cellular level. In static cultures of the Maximow type, however, the normal epithelium, in the presence of connective tissue, organizes in tubes and alveoli, whereas in the same conditions the neoplastic epithelium does not. In roller tubes, the behavior of both epithelia is identical; they both terminate in loose cells comparable to the HeLa cells. When the milk agent is introduced into normal cultures, one can only notice a slight growth activation. In such conditions the detection of the virus is difficult. Bioassays are made as a routine procedure, but, in this particular case, results cannot be expected before 12 to 18 months. New methods are needed. The fluorescent antibody technique developed by Coons is, in our opinion, worthy of special attention. With labeled γ-globulin, Weller was able to study, direct from tissue-culture preparations, the cell-virus relationship of varicella and herpes zoster; Coffin and Cabasso studied infectious canine hepatitis; Jacqueline Lebrun, herpes simplex. We, personally, made 2 preliminary attempts to follow the fate of the milk agent in our cultures, and we have strong indications of the applicability of this method. With cultures of a mammary carcinoma, we observed a marked fluorescence of the cell boundaries that gave the appearance of a honeycomb structure to the preparation. In cultures of mouse epithelium inoculated with the agent, the fluorescence was rather localized in the cytoplasm, thus suggesting that the agent is first synthesized in the cell, then migrates to its surface where it is released in the milk or the culture medium. Electron microscopy confirms some of these findings by showing the presence of a dense population of virus-like particles 100 mμ in diameter, which is located in the intercellular spaces of mammary carcinoma cultures. Later, identical bodies were found as free particles in the medium itself. If they actually represented the inducing agent responsible for the carcinogenesis, the fluorescent-antibody technique gave quite accurate results. Quantitative information can probably be gained from the adjustment of this method, and, if so, we would have a convenient way of testing any viral agent in culture with corresponding stocks of labeled γ-globulin.

Dr. Weller: Dr. Syverton has presented an admirable summary of advances in virology as influenced by the utilization of tissue-culture procedures. He has indicated the general indebtedness of virologists to those workers responsible for the development of tissue culture as a science. At this time, it is a privilege to acknowl-

edge a continued indebtedness to the Tissue Culture Association for its program of media certification and its magnificent bibliography, and to the individual members who have so often assisted us with our technical problems. In Boston, for example, Dr. John Hanks has given generously of his time on innumerable occasions.

The discussants have been charged with "opening up questions." Within the framework of Dr. Syverton's presentation, there is little space to raise questions of fact or of interpretation. Indeed, the only point that I would like to raise is one of semantics that deals with a connotation implied in the use of the phrase "stable cell strain." In view of the discussions of this Conference, I feel that we are justified in considering a cell strain, particularly after a period of cultivation, as being composed of a mixed population that may manifest variability in one or more capacities including that of being able to propagate virus. Certainly there is no objection to the term "stable cell strain" if one uses it in a relative sense. Yet some of the present difficulties in the field of virology appear to arise from the fact that a so-called "stable cell strain," as maintained in one laboratory, supports growth of a particular virus, whereas the same strain, when transferred to a different laboratory, lacks this capacity. To be sure, such discrepancies are often due to extrinsic rather than intrinsic factors.

Next, I would like to comment on the selection of specific techniques as applied to the study of viral entities. The virologist selects an *in vitro* technique that is suitable for his experimental objective. This selection obviously is crucial and at the moment is often an educated guess. Many variables must be considered. The host cell population not only must be capable of being infected by the agent under study but should respond with gross metabolic or anatomic alterations in such a manner that the damage becomes overt. The medium chosen to maintain the cell population must provide a level of cellular metabolic activity sufficient for the virus to propagate. Components of biological origin in the experimental system should be recognized as potential sources of extraneous viral agents that may invalidate the results. Furthermore, the medium may *per se* inactivate the agent under study. To compound difficulties, the virologist must consider that the virus population under study may be extremely variable and may contain elements capable of growing *in vitro* without producing cytopathic changes or may persist in latent form through many tissue-culture passages. At present, we know little of these variable factors. A few examples will indicate certain problems.

As you know, poliomyelitis virus grows well in cells of monkey and human origin under the simplest of cultural conditions. Indeed, as shown early and recently documented biochemically by Eagle and Habel, the requirements for synthesis of poliovirus in a susceptible cell may be independent of the requirements for cellular growth. Yet, while the rhesus kidney cell readily supports the growth of poliovirus *in vitro*, Melnick observed that in the capuchin monkey kidney cell cultures, overt cytopathogenicity is lacking, and only a small percentage of these cells appear to support viral proliferation. Again, as reported by Ackermann, certain HeLa cells are not destroyed by poliovirus but persist with altered characteristics.

At the other extreme, certain agents manifest cytopathic activity *in vitro* only under conditions permitting excellent cellular growth. The viruses that cause varicella and herpes zoster in man and the recently isolated agent of cytomegalic inclusion disease appear to fall in this category. The varicella-zoster agents and the cytomegalic agent have much in common. They belong to the group that produces focal cytopathic changes and regularly induces the formation of intranuclear inclusion bodies *in vitro*. In culture the foci enlarge under conditions that suggest a direct transfer of infectious material from cell to contiguous cell. In the varicella agent, at no time during prolonged serial cultivation have we been able to demonstrate the presence of infectious material in the fluid phase of the culture. It is of considerable interest that the morphology of the cell colony *per se* influences the rate of propagation of the varicella agent. If the cell network is loose, viral progression from cell to cell appears to take place slowly. Conversely, in a densely packed mass of tissue this transfer occurs more

rapidly. If the culture system is altered in the direction of impaired cellular activity, viral proliferation, as evidenced by increase in size of the focal lesion, is either slowed or stopped completely. That the agent transfers from cell to cell is further suggested by the fact that this process continues in the presence of homologous antibody incorporated as a constituent of the medium.

In conclusion, I should like to stress that we lack the basic information necessary for an informed selection of suitable *in vitro* techniques. As Dr. Lwoff pointed out, the virologist utilizes tissue culture to kill cells. At the moment, the virologist is, perhaps, comparable to the mortician. Most frequently, he is satisfied with observing that the patient is dead and discards it in the crematory. On occasion he may embalm it and preserve it in a suitably stained environment. In the future, we hope that the virologist will assume the role of a cell physician seeking to detect and understand subtle metabolic and anatomical alterations induced by the parasitic agent in the infected cell.

Dr. Harris: I wonder whether I could needle Dr. Syverton gently on one statement: that established strains properly replace the freshly isolated cell. Perhaps the sweep of current research will force us to modify this idea. The nutritional qualities of freshly isolated cells may differ from those of established strains. I wonder whether similar differences may not exist in reactions to virus. On the other hand, it is a fact that, with improved methods, one can obtain reproducible growth, at least with chick cells directly following isolation from the embryo. Even clones can probably be obtained at this stage. I wonder, therefore, whether it is the freshly isolated condition as such or the instability of fresh preparations which you had in mind when making this statement?

Dr. Dunnebacke Dixon: We have just recently encountered the problem of primary cells *versus* passaged cells, and I would like to tell you what has happened so far. We have tried to get cultures of amnion into serial cultivation. We noticed that between passages 5 and 9, the cultures changed from the normal epithelial type of cell to a very much enlarged fibroblast-like cell. When we infected these enlarged fibroblastic cells with poliovirus, we found that they no longer reacted like the primary amnion cells with the loss of the nucleolus and the formation of the nodules. Instead, the passaged cells underwent changes similar to those observed in HeLa and monkey kidney cells. We feel that something certainly has happened during the period of cultivation which has affected not only the gross morphology of the cells but also the cytopathic expression of poliovirus infection.

Dr. Weller: I think perhaps the objections will be resolved when Dr. Syverton and others standardize the newer techniques of freezing and preserving cell strains. Then we will have a reference standard which we can refer to. But certain considerations regarding the use of cell lines for isolation of unknown viruses are important. It is obvious that the easy viruses have now been isolated, including many of the important pathogens. We are looking for the elusive viruses. And, to find them, it has seemed to us that our chances would be enhanced if we took cells directly from the normal host, preferably employing a mixed population of cell types to augment possibilities of finding some susceptible cell.

Dr. Chèvremont: In regard to the possible changes resulting from carrying the cells for a long time *in vitro*, I should like to recall some of our observations. You know that many fibroblasts and myoblasts can be turned spontaneously into macrophages and that a pure chemical substance, choline, can promote this transformation when the cells are taken directly from the organism; but when they have been transplanted 4 or 5 times, this spontaneous transformation into macrophages disappears; also after 5 transplantations, and more, choline is no longer active. I think something must have disappeared in the chemical composition of the cell and probably in the metabolic properties.

Dr. Hull: Dr. Harris has raised the question of the relative merits of primary cells *versus* stable cell strains. We have isolated 25 or 30 simian viruses and have two that will not grow in a stable monkey kidney cell strain but can be cultivated readily in primary monkey kidney cells.

I approve Dr. Syverton's emphasis on the use of cytopathology as a valuable technique for the virologist. In the course of isolating these 25 or 30 viruses, we have used cytopathology to great advantage as an aid in putting them in groups for the purpose of identification. We originally placed these viruses in 4 cytopathological groups. Most of our 4 groups could be integrated into Dr. Syverton's 5 groups. Later, we found that of the similarities and dissimilarities, which one might find by serological studies, by means of neutralization tests and other serological studies, such as hemagglutination and complement fixation, virulence studies, inactivation, etc., the similarities were generally within one CPE group—the dissimilarities between the groups. This showed that the original grouping on the basis of the type of cytopathology produced appeared to represent good criteria for characterizing or classifying this group of agents.

Dr. Dunnebacke Dixon: It might well be secondary. I have no idea what happens before. Dr. Lumsden might comment on his observations on the nucleolus.

Dr. Lumsden: I do not have very much to say to that except what I reported to the Tissue Culture Association meeting in Milwaukee (Lumsden, C. E.: Effects of antibodies on cells in tissue culture. Anat. Rec. 124: 17, 1956) on the effects of heterospecific antibodies on cells at high titers. One point I made, was that these effects are rapid and appear to be concerned primarily with the cell membrane and the ground substance of the cytoplasm. Also, even within a few minutes, progressive changes begin in the nucleolus and nucleus, with extrusion of substances from them into the cytoplasm. Personally, I think a great deal more work should be done on the processes of autolysis of dying cells before we attempt to draw conclusions about the specificity of any changes seen in cultures infected with viruses.

Dr. Duryee: It might help us to interpret some of the beautiful preparations that we saw on the screen if we think for a moment in terms of nuclear physiology. In our laboratory we have been studying these problems for a number of years, and we have seen, in many kinds of cells both normal and malignant, a process that seems to be quite fundamental; namely, the nucleolus pulsates and discontinuously swells, bursts, and is extruded into the cytoplasm. We think this is a normal method by which RNA is put out into the cytoplasm to serve as a template for proteins. When one infects frog kidney tubules with a filtrate from a tumor containing a presumable virus, the first appearance is a new DNA within the nucleolus of some of the host cells. The nucleolus as Dr. Syverton showed may become swollen with these large inclusion bodies, as similarly described by Dr. Lucké years ago. In more malignant states they occasionally fill the whole nucleus. Sometimes they are retained within the nucleus or they may be extruded into the cytoplasm to make the kind of cytoplasmic inclusions that Dr. Weller and others showed here.

Dr. Gey: I think we should be a little more fastidious in our laboratory procedures and especially in our interpretation of processes where viruses are involved. First of all, we should define and keep in mind constantly the ratio of virus to the cellular population, with emphasis on the infectious units. In this regard we have tried to perfect our centrifuge method, which allows for the segregation of all of the dispersed virus onto cultured cells. We have demonstrated that this can be done by applying high gravitational forces to the cultures. Why is this important? Because, if you follow very carefully the response of cells to viral infection, you will see a whole series of changes. Recognition of these changes is exceedingly important to an understanding of new virus production and cell response. The centrifuge methods provide an approach that is helpful if one is trying to classify the responses of cell strains, or with respect to virus or even to the quality of viruses in a given preparation. In a routine procedure, if a virus like that of Eastern equine encephalitis is added to a roller tube of HeLa cells, you will expect to see some changes in the first cells exposed. We thus need to establish a zero time of contact for a given cell under observation. There is reason to believe that this cell may disintegrate and then donate virus to other cells not previously exposed. The product of this destruction may be either highly infectious virus or incomplete virus and, in addition, dead virus and cell remnants. From these we may assume a second wave of infection or exposure. This wave

of infection, if it exists, is again variable. One can say at least that a dispersed virus is produced. There are other features of interest. During the process of infection, when many cells are being destroyed, some cultures, by means of phagocytosis, may show an incorporation of fragments or of whole dead cells or of degenerating cells by members of the surviving population. Even dividing cells may be found to incorporate dead or partially degenerating cells, which may be considered here as infectious units of the virus population. How did these cells become resistant during the infection; or are they resistant members of the population? It seems to me that in all of this progression of infection one must consider the virus in all its aspects, the cell in all its aspects, and the many stages in which the cell and virus, as a class, may exist.

Dr. Reissig: In studies in which the growth cycle of poliovirus is correlated with the time of appearance of cytopathic changes, virus production is often measured in terms of the increase in the infective titer of the culture fluid. However, results of work done in our laboratory, in collaboration with David Howes and Dr. Melnick, have shown that in the case of poliovirus multiplying in monkey kidney monolayer

PLATE 61

All figures represent photomicrographs of monkey kidney epithelial cultures fixed in Zenker's fluid and stained with hematoxylin and eosin. Key to figures: *Nm*=nuclear membrane; *n*=nucleolus; *Ip*=intranuclear inclusion typical of poliovirus; *Im*=intranuclear inclusion typical of measles virus; *Cm*=measles type of cytoplasmic inclusion; *Cp*=cytoplasmic mass characteristic of poliovirus.

FIGURE 1.—Monkey kidney culture 4 hours after infection with poliovirus. The 2 cells at the *right* have small eosinophilic intranuclear inclusions. The cell at *upper left* appears normal. Approx. × 1,000

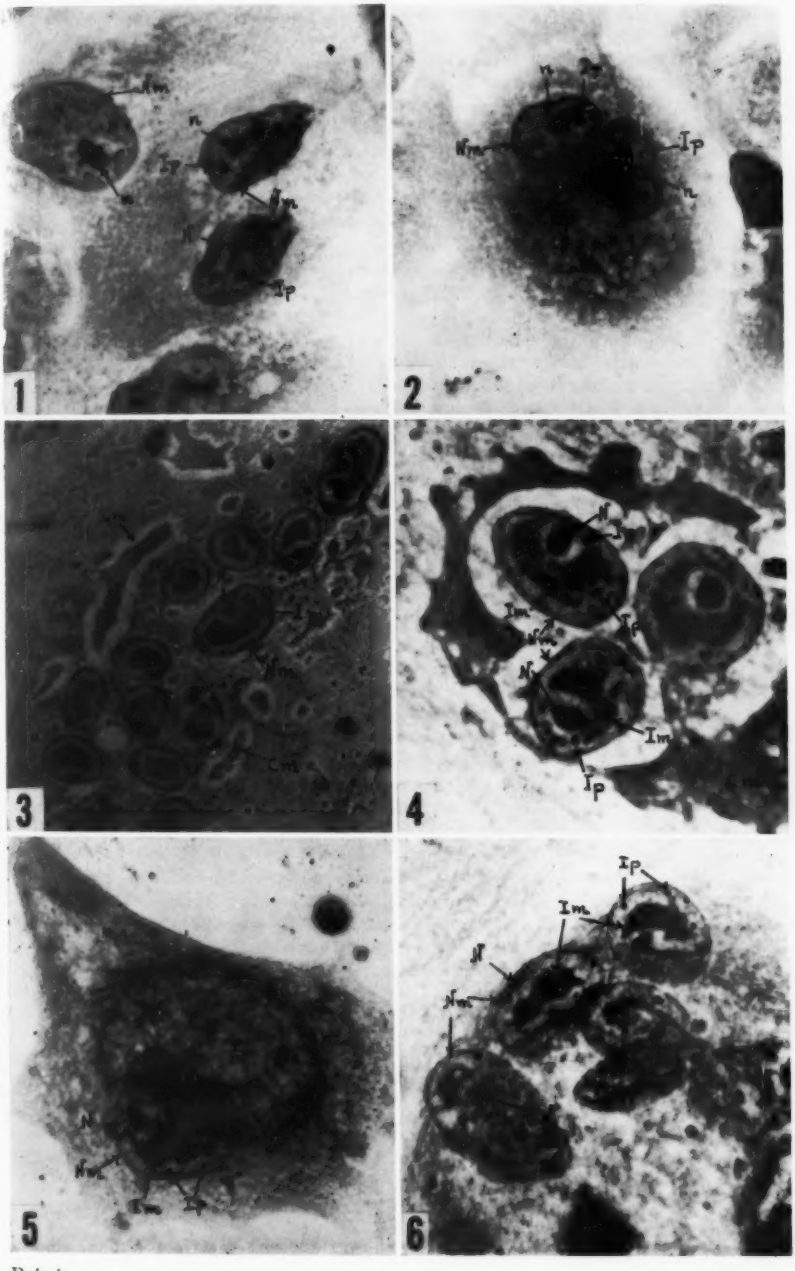
FIGURE 2.—Cells from a similar culture in a slightly more advanced stage of cytopathic degeneration. Two small eosinophilic intranuclear inclusions and a cytoplasmic eosinophilic mass. Approx. × 1,000

FIGURE 3.—Multinucleated cell, typical of measles virus infection in monkey kidney cultures. Both cytoplasmic and large intranuclear inclusions are present. Approx. × 500

FIGURE 4.—Monkey kidney culture inoculated with measles virus and superinfected with poliovirus several days later. Three nuclei from a multinucleated cell are seen, partially surrounded by a cytoplasmic inclusion of the type seen after measles virus infection. The nuclei contain both the large, light-red inclusions typical of measles infection, together with several darker and smaller inclusions of the type usually found in poliovirus infected cells. Approx. × 1,000

FIGURE 5.—Another field from the culture shown in figure 4, showing a mononucleated cell containing both types of intranuclear inclusions and also a cytoplasmic mass characteristic of poliovirus infection. Approx. × 1,000

FIGURE 6.—Another field of the same culture showing a multinucleated cell containing both types of intranuclear inclusions. Approx. × 1,000



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cultures, extracellular virus titers do not give a true picture of the total amount of virus produced and, particularly, of the time when it first appears. In this system, new infective virus appears within the cells from 1 to 2 hours or more before it is released into the fluid, and the intracellular virus titers are much higher than the extracellular ones. When single cell growth-curve experiments were performed, new intracellular virus appeared as early as 3½ hours after virus inoculation, several hours before the cells rounded up, and the cytopathic effect could be detected in unstained cultures with the ordinary microscope. However, when these cells were fixed and stained with hematoxylin and eosin, virus-induced specific changes were seen as early as 3 to 4 hours after infection. These early changes can be seen in figure 1, plate 61. They consist in the appearance of eosinophilic intranuclear inclusions surrounded by a clear halo, loss of the normal chromatin network, and wrinkling of the nuclear membrane. Some time later, eosinophilic cytoplasmic masses appear (fig. 2, plate 61), and the cells begin to pull away from the glass. This retraction of the cell boundaries is the first definite change that can be distinguished with the phase microscope in living cultures. Thus, fixation and staining of the poliovirus infected cultures enables us to detect the cytopathic effect earlier in a larger proportion of the cells (*see* J. Exper. Med. 104: 289, 1956 for details).

Monkey kidney cells, in culture, that have been infected with measles virus and are actively producing it remain susceptible to poliovirus. The cytopathic changes induced by measles virus are characterized by the appearance of eosinophilic intranuclear and cytoplasmic inclusions and formation of multinucleated cells by fusion of mononucleated ones. As seen in figure 3, these changes are quite different from those induced by poliovirus. Morphological evidence was obtained, which suggested that infection of 1 cell with both viruses might occur. In these doubly infected cultures, both mononucleated and multinucleated cells possessing intranuclear and cytoplasmic inclusions characteristic of measles virus also exhibited, at times, paranuclear eosinophilic masses or intranuclear inclusions typical of poliovirus alone (figs. 4, 5, 6, plate 61). No significant difference was found in the poliovirus yields from cultures previously infected with measles virus as compared with those infected with poliovirus alone.

Dr. Bang: In using the electron microscope, I was trying to emphasize the need for understanding and unraveling the pathogenesis of individual virus-cell relationships. I think the studies that Dr. Reissig has described are certainly the right approach. What I meant by saying there is a startling lack of data is, for instance, brought out here. Does the virus start in at the surface, even though one sees very little with the ordinary microscopic preparation? Are mitochondria changed? There are excellent movies, as we have seen, on a number of cases of mitochondrial movement. As far as I know, nobody has made an attempt to find out whether mitochondria are altered in any way, in the initial stages of a virus infection, so that one might think of the actual carriage of the virus bodies. This approach can be used in studying the nucleus, and we can then discuss whether the nuclear changes are primary or secondary. I think each one of these problems will vary with the specific infection. The great advantage we have today, with some sort of uniformity of cell populations, is that we can now begin to study these processes.

Dr. Willmer: While it obviously cannot provide a complete answer, I wonder whether there is any relationship between focal infection and occurrence of mitoses, because there is some evidence that the growth-promoting substances (if I may use the word again) get into the cell more easily, either at or immediately following cell division. Because certain of these growth substances may be of a nucleoprotein character and therefore in some ways similar to viruses, the focal infections might perhaps originate more easily from cells that were dividing or have just divided.

Dr. Crocker: The advantages of phase-contrast microscopy for study of virus infections in cells were well described by Dr. Bang. Light microscopy cannot resolve particles of the size of most animal viruses, and it is insufficient for observation of cell organelles as small as most components of the ergastoplasm. However, structures too small to be seen as individuals may become "visible" when aggregation or other

changes occur in their distribution within living cells. If these alterations are recorded by time-lapse photography, the value of phase-contrast microscopy is greatly enhanced due to the availability of movement as an index of location and rate of reactions. Thus, some early cellular responses to infection or injury may be recognized by altered movement and activity among small structures in a portion of the cell; such effects might be missed upon microscopy without time-lapse records.

This method has been applied in virus studies, as noted by Dr. Bang. Micrography with phase-contrast optics and by standard optics with stained material has been referred to here by Dr. Dixon and by Dr. Reissig and Dr. Melnick. This evening, we will show a time-lapse record of infection of HeLa cells by a virus of the psittacosis group. In this record there is little evidence of abnormal cell activity or structure except in the inclusion that becomes very large during massive viral proliferation. Such slight harm to infected cells suggests that this virus grows with little demand on the cell and interferes very slightly with those cellular resources that are involved in motility, nuclear oscillation, movements of cytoplasmic edges, pinocytosis, and structural integrity of mitochondria. In contrast to the limited early injury produced in cells by psittacosis viruses, rapidly changing cell responses occur in cells infected with poliomyelitis virus. This corresponds with rates of propagation of the two viruses, of course, but local cellular changes differ in the two infections as well. Observations of this type should aid in defining cellular organelles and, from these, metabolic sites or systems, upon which viral reproduction depends.

Dr. Syverton:—I should like to suggest for comment several points of concern. First, over a period of 22 years, I have searched stained tissue and cell preparation for evidence that the processes of mitosis and production of a virus inclusion body can occur concurrently in a single cell. I have not found such evidence. It should be interesting to learn from the collective observations of this group whether my experience is common to other persons in the field. It is of interest that we can irradiate cells with total stoppage of mitosis, yet the irradiated cells produce inclusion bodies and as much virus as normal cells. This latter experience may influence your comments.

Second, it was common experience until recent years to employ mixed cell cultures. For example, Dr. Parker and Dr. Carrel maintained chick cultures of mixed cellular origin for many generations over many years. It is difficult, if not impossible, with a mixed cell population to employ the present-day quantitative approaches for assessing response of single cell types to virus infection. There is, of course, the approach for quantitating virus by employing plaque-count technique, which permits assay of a number of virus particles to infect and thereby to evaluate multiplicity of infection and to relate "burst" to other phenomena.

Pathology in Fields Collateral to Tissue Culture [1,2]

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The temptation to interpret the title of this section as "The Pathological Nature of Tissue Culture and Tissue Culturists" has been resisted. In retrospect, it is apparent that my confusion with reference to the title arose from the fact that tissue culture is referred to as a field. I had always thought of tissue culture not as a field but rather as a tool for cultivating a field. A tool which in some hands is used as a plow, in others as a harrow, and in still others, I think, as a manure spreader. I am still not entirely clear as to the meaning of the title, but inasmuch as I am a pathologist with a primary interest in animal transplantation, I have adopted the implication that my purpose is to discuss an approach by means of animal transplantation to some problems in disease.

Animal transplantation offers an approach to a number of important problems in the 2 main subdivisions of pathology—neoplasia and inflammation. In both, the techniques employed, unlike those of tissue culture, are largely concerned with establishing a vascular union between transplant and host. Such a union is often facilitated by employing specific regions of the body, notably the anterior chamber of the eye; and some preliminary discussion of the methods utilized may be of value before proceeding.

The technique of anterior-chamber transfer is extraordinarily simple. The animal is immobilized with tie strings on an operating board in such a manner that the desired eye is available to the operator. The cornea is anesthetized by contact with a 5 percent aqueous solution of cocaine administered with a medicine dropper. An incision is made at approximately the midpoint of the upper border of the limbus with a sharp, double-edged corneal knife. The knife is directed slightly forward so that the blade enters the anterior chamber without damage to the iris. The pressure necessary to pierce the cornea is sufficient, even with a sharp knife, to rotate the eyeball beneath the lower lid and conceal the operative field. A short, quick thrust of the knife, however, results in an adequate opening and the temporary obscurity is inconsequential. With-

[1] Received for publication May 28, 1957.
[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8–12, 1956.

drawal of the knife is accompanied by the escape of a small amount of aqueous humor, but, unless the iris has been cut by a misdirected knife, there is no bleeding.

Transfer of the tissue is effected by means of a trocar commensurate in diameter with the corneal incision. The trocar should be equipped with a tight-fitting plunger and a short beveled mouth with all edges filed to smoothness. A small fragment of tissue is placed in the mouth of this trocar and forced into the barrel, a step greatly facilitated by withdrawing the plunger to produce suction. The tip of the trocar is inserted through the incision a short distance so that the bore is entirely within the chamber, and the fragment is expelled. It is important that all manual pressure about the eye and the head of the animal be released before withdrawing the trocar to prevent the escape of the fragment. Finally, the fragment is forced into the inferior angle of the iris by applying light pressure along the corneal surface with a blunt instrument. This incision is not covered.

The procedure is executed with rapidity and the technique is extremely simple, yet several points require further emphasis. The corneal knife should be sharp and of such width that a stab wound will admit the trocar. The use of a narrow knife necessitates side cutting to obtain a sufficient opening and this may be attended by iris damage with consequent bleeding. Pointed surgical blades or single-edged corneal knives are undesirable, for the triangular cut produced heals slowly and may result in herniation of the iris.

The fragments of tissue to be transplanted should be cut from the mass with sharp instruments and should not exceed 1 mm. in diameter. The placing of the fragment in a wedged position in the inferior angle of the iris is important for early vascularization. Occasionally, these fragments work loose shortly after operation, and an examination, with required readjustment, is desirable before returning the animal to the colony. Fixation occurs within a few hours, and further check is unnecessary.

The tissue may be transplanted immediately after removal or stored at refrigerator temperature for several days. In the latter case, it should be kept free of contact with hospital physiological saline or other noxious fluids. It is essential that the material be free of infection and that surgical sterility be maintained throughout all manipulations.

The brain and the eye have been used extensively in the investigation of tumors, particularly, of the development of tumors, and this phase of the work may be pertinent to the present meeting inasmuch as tissue-culture studies may offer a means of approach to several primary problems.

The point of significance is that cancer is not a sudden transformation of normal cells but, on the contrary, represents the final step in a developmental process. It is curious that the former concept is still widely held despite an abundance of contrary evidence derived both from experimentation and simple observation. It is felt that, like the phoenix, cancer arises full-fledged with all of its malignant properties in instant operation.

This is most emphatically not the case—the distinguishing attributes of cancer are not present from its initiation but represent developmental acquisitions.

One of the most impressive biological attributes of cancer is its ability to metastasize. The growing tumor invades blood or lymph channels, and cells are swept off into the fluid stream and transported throughout the body. They lodge in various organs, divide inside of the vessel for awhile, then attain a separate blood supply and grow into adjacent tissues. The secondary growths thus formed are metastases and constitute the preeminent cause of death in cancer patients.

It is important to emphasize that the ability to metastasize is not a property of the tumor from its conception. On the contrary, it is a developmental acquisition. During most of its course, the cells of the tumor, although indistinguishable in appearance from those found in late stages, do not possess this property. Attainment of this ability is a function of continued development and, unfortunately, is not associated with any recognizable change in the microscopic structure of the growth. This phase is of relatively short duration and is terminated by the death of the individual.

Although the ability to metastasize is not reflected in microscopic appearance, a coincident manifestation of autonomy offers a laboratory method by means of which the developmental stage of the tumor can be determined. This test is based on behavior and involves transplantation. Prior to the development of metastasizability, the tumor can only be successfully transplanted back into the individual in which it arose or to other individuals bearing similar growths. It is dependent for its continued existence on factors peculiar to the tumor-bearing individual. Such factors are not supplied by normal individuals, and the tumor will not survive transplantation to them. Coincident with the attainment of metastasizability, the tumor becomes independent of these factors, or autonomous, and will grow in their absence. At this stage, it can be readily transplanted to normal unrelated individuals and species is no barrier. Thus, human tumors that have reached this phase of development can be transplanted to guinea pigs or mice by utilizing suitable techniques. Failure to survive such a transfer means that the tumor in the patient has not yet developed to the point where metastasis is possible, whereas growth in the foreign species means that it has attained this ability (figs. 1, 2, 3, 4, plate 62).

Comparatively little is known about the factors concerned in dependency except that they are present from the earliest stages of tumor development and, with the exception of the virus-induced tumors, are systemic or constitutional in nature.

The transplantation situation with respect to the Shope papilloma and the Rous sarcoma is quite different. Both of these tumors possess the ability to survive and grow on homologous and heterologous transfer to normal animals from their earliest stages of development. Transplantability is present from inception, yet metastasizability is only acquired after an

extended developmental course. Investigation suggests that the intracellular virus is analogous in its operation to the constitutional factors concerned in the dependence of other tumors and that the transplantability and growth of the Shope papilloma and the Rous sarcoma in normal animals is conditioned on the concomitant transfer of their dependent factors.

A conception of the virus as a conditioning factor analogous in its action to the constitutional factors concerned in the dependence of other tumors and located in the cell rather than in the host implies that transfer of an infected cell would necessarily be accompanied by transfer of the factor on which its growth depends. Thus, the special environmental conditions required for the growth of other tumors transplanted during dependent phases of development are replaced by intracellular components in virus tumors and, in this respect, the survival and growth of transplants are independent of the constitutional status of the host.

Despite the paucity of information, it is apparent that the factors governing the development of a tumor are of the utmost importance in cancer research. Our investigations have been concerned with attempts to supply the missing factors by an experimental modification of normal animals and thus to institute the necessary conditions for growth of dependent tumors. A hampering problem in this study has been the maintenance of a source of dependent tumor tissue, for, by its nature, dependent tumor tissue will not grow in normal animals. It would appear that tissue culture might offer a much more rapid means of advance. If dependent tumor tissue could be grown in culture media, a constant supply of material would become available. Further, modification of the nutrient environment of the culture might be tested by subsequent animal transfer to determine possible effects on the development of autonomy.

It is a point of some interest that the ability to grow in unrelated animals and species is not limited to cancer tissue but is shared by embryonic tissue. Adult tissue will not survive heterologous transfer but grows readily on homologous transfer to the eye or the brain. The growths resulting from the homologous transfer of adult tissue are true transplants and are not merely surviving tissues as they continue to function in some cases and are susceptible to infection in others. The ability to grow adult tissues in related animals of different constitutional type allows a unique approach to the problem of susceptibility and resistance to infection.

For example, the Shope papilloma is a virus disease of rabbit skin. If fragments of adult rabbit skin are cultured overnight in the presence of this virus and then transplanted to other rabbits, the typical papillomatous lesion occurs in the transplant. It is significant, immunologically, that the papilloma develops whether the skin used for transfer was derived from an immune animal or whether the new host itself was immune to the virus. These experiments can be carried over to resistant species by utilizing embryonic tissues infected *in vitro*, and it would seem to me that

further studies of this nature combining tissue culture and animal transplantation might be highly productive.

Embryonic tissues grow and mature into adult functioning structures on both homologous and heterologous transfer (figs. 5, 6, 7, 8, plate 63). Embryonic rabbit skin is extremely susceptible to infection with the Shope papilloma virus, and papillomas develop in homologous transplants irrespective of the immunological status of the adult host. The high susceptibility of embryonic rabbit skin suggested the possibility that embryonic tissues of naturally resistant species might also be susceptible. This was true in the rat—a species completely resistant to the virus, and embryonic rat skin infected in culture with the virus and transplanted to adult rats developed the typical Shope lesion.

The susceptibility of embryonic tissue to infection with agents to which the adult of the species is naturally resistant requires further exploration. Preliminary studies have shown that susceptibility is not limited to the Shope virus but includes agents of human disease that have not been successfully transferred to laboratory animals. Notable in this respect is a disease in man known as Boeck's sarcoid. This is a granulomatous disorder somewhat resembling tuberculosis. Many of its features suggest an infectious disease, but it never has been reproduced in laboratory animals. However, fragments of embryonic mouse thymus cultured in the presence of a Berkefeld filtrate of a human lesion, and subsequently transplanted to adult mice, show a morphological picture identical with that of the disease in man.

Embryonic tissues are also susceptible to heterologous bacterial infections. Leprosy is a case in point. Hansen's bacillus has not been successfully transmitted to laboratory animals. Yet the bacillus survives in transplants of embryonic guinea-pig dermis and has been carried for 8 generations by serial transfer.

Unlike adult tissue, embryonic tissues will also survive and grow on heterologous transfer, and this ability further enhances the value of the technique for infectious disease study (figs. 9, 10, 11, plate 64).

Embryonic tissues of susceptible species infected in culture and transplanted to naturally resistant species also respond with the development of the typical lesion. Embryonic rabbit skin infected with the Shope virus and transplanted to hamsters, guinea pigs, rats, etc., develops the lesion characteristic of the disease. Embryonic rabbit connective tissue exposed *in vitro* to the fibroma virus reacts similarly on heterologous transfer and the disease is reproduced in the transplant. The transplantability of embryonic tissues transcends the barriers between zoological classes, and it is thus possible to induce chicken diseases in mammals and *vice versa*. Embryonic chicken skin infected with fowl pox and transplanted to mice develops the characteristic disease picture. Perhaps of greater interest is the fact that embryonic chicken connective tissue infected with the Rous virus, and transplanted to mammals gives rise to the Rous sarcoma (fig. 12, plate 64).

These are only a few examples of the use of animal transplantation in

the study of disease, and many others must be apparent. It is suggested that in a number of cases, the combination of tissue-culture techniques with those of transplantation might offer a more promising approach.

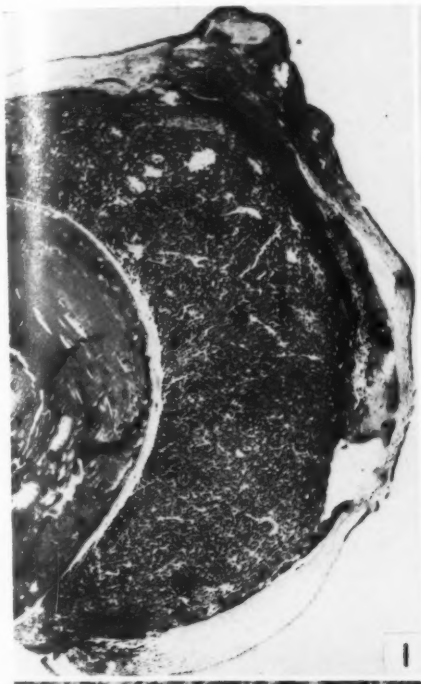
PLATE 62

FIGURE 1.—Brown-Pearce rabbit cancer in eye of a mouse. The anterior chamber is filled with growing tumor.

FIGURE 2.—Human glioblastoma in brain of a mouse. The darker portion is the growing tumor.

FIGURE 3.—Human fibrosarcoma growing in the anterior chamber of a guinea pig's eye.

FIGURE 4.—Human papillary carcinoma of the thyroid growing in a guinea pig's eye.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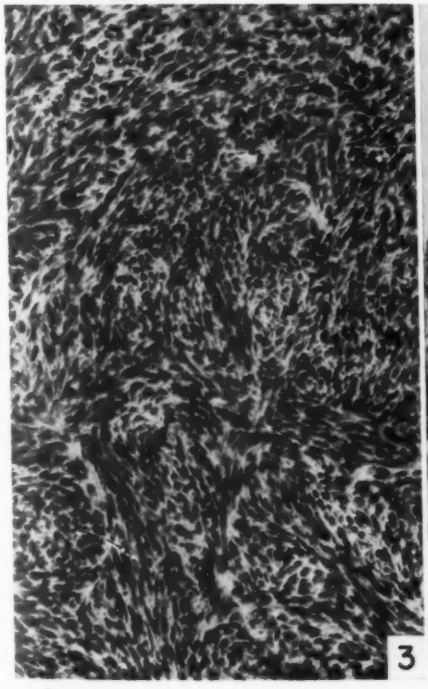
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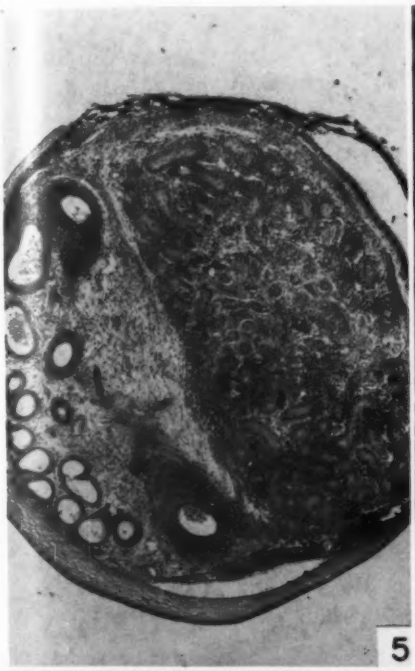
PLATE 63

FIGURE 5.—Embryonic mouse testicle and epididymis growing in eye of adult mouse.

FIGURE 6.—Embryonic guinea-pig intestine growing in brain of adult pig.

FIGURE 7.—Embryonic mouse brain growing in brain of adult mouse.

FIGURE 8.—Embryonic guinea-pig kidney growing in brain of adult pig.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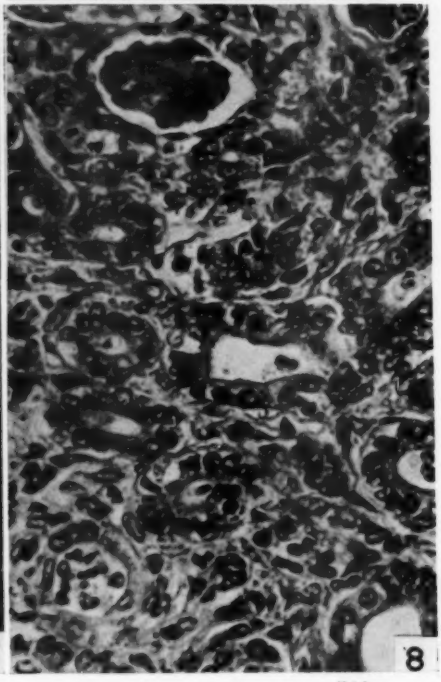
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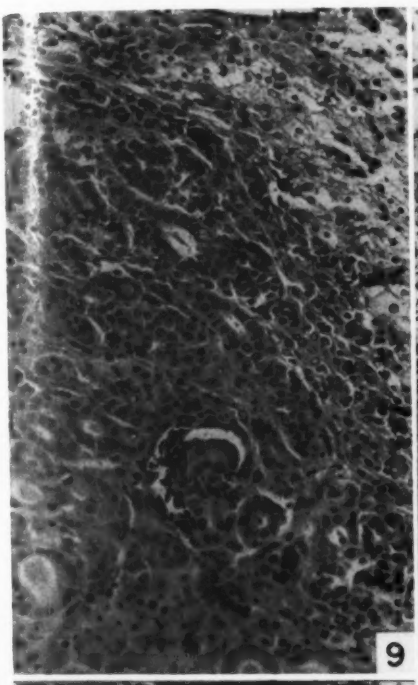
PLATE 64

FIGURE 9.—Embryonic mouse kidney growing in brain of a guinea pig.

FIGURE 10.—Embryonic human lung growing in anterior chamber of guinea pig's eye.

FIGURE 11.—Embryonic human intestine growing in anterior chamber of guinea pig's eye.

FIGURE 12.—Rous sarcoma arising from transplant of infected embryonic chicken tissue growing in the brain of a mouse.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DISCUSSION

Dr. Hyatt: Dr. Greene's presentation of cell personality that becomes paranoid through development and then overreaches normal anatomical boundaries to the point of destruction of the functional and biological communities is in keeping with clinical observations.

As an orthopedic surgeon who finds that his treatment ideal is limited to considered mutilation, varying from amputation, to the palliative but usually hopeless *beta* and *gamma* irradiation, the problem is critical in many, many ways. In the light of this, I should like to review briefly my version of Dr. Greene's concepts. Dr. Greene states that the failure of a tumor from a human being to survive transplantations to a foreign species, means that the primary human tumor tissue has not developed a *metastasizing ability*. If this concept is to be clinically significant, and certainly Dr. Greene's slides indicate that it is, it behooves us to approach the problem on a greater scale and run the gamut of the potential oncogenic tissue types. It is important to determine, through human autopsy, whether the tissue tested represented a primary tumor or a metastasis, and to accumulate *statistically validated quantitated* data on this question. However, a point that bears emphasis at this time is that more people die from the accumulative wear and tear of old age than from malignancy, and, with the current deification of the automobile as a rapid and relatively uninhibited medium of transportation, the acute wear and tear on the human body is devastating. The resulting demand for anatomical parts, for suitable replacement of the injured, defective, or destroyed parts, is becoming increasingly significant.

As one of those most responsible for the development and current function of the Tissue Bank at the National Naval Medical Center, I should like to explain our specialized interest from the point of view of the potentialities of tissue culture. First, however, I should like to acknowledge our debt to tissue culture. The work of Hanks and his use of the balanced salt solution and 10 percent serum as media for skin stored at household refrigerated temperatures, encouraged us, originally, and sustains our interest in tissue viability and its storage today. The advice of Earle, Evans, and their associates at the Tissue Culture Section in the Laboratory of Biology, National Cancer Institute, assisted us materially in the original design. They subsequently provided training and laboratory hospitality that to us, as surgeons, has been incomparable. We are looking forward to the future with keen anticipation largely because of the nature of this collaboration; we hope to lean heavily on Algire and Merwin, and to follow the example of Gaillard in the practicality of clinical applications. While tissue culture has certainly provided us with assistance in the maintenance and variability of cellular components of human grafts, we must admit that the majority of the reparative and reconstructive surgical contributions to date have depended, in much greater part, on the preservation and utilization of the cellular matrix. Bone, fascia, dura, artery, and skin are offered as examples of this concept. Viability of the cellular component associated with these matrices is unnecessary and, in fact, it may be disadvantageous in the adult homograft. When we deal with the adult homograft that is to be transplanted to the well-vascularized operative site, this graft, with few exceptions, is clinically successful, because it is biologically acceptable in the operative site while performing primarily a mechanical function. It induces the host to revascularize the graft and provides a framework in the form of the graft. Revascularization is associated with resorption of the cellular matrix and replacement by matrix of host origin. During the replacement, satisfactory mechanical function is maintained so that ultimately the components of the host assume all of the mechanical and the metabolic demands of the operative site, yet no vestige of the original homograft

remains. An instance may be cited. When a blood-vessel homograft segment is required to bridge a gap in the large blood vessel of a patient, the elastic fibrillar network of the graft is most responsible for clinical success. These elastic fibrils gradually wear out and are not replaced by elastic fibrils from the host, though the functioning of components of the elastic network have been observed as late as 1 year after transplantation into the adult. The host fibroblast is ultimately associated with the formation of enough collagen fibrils to form a functional although relatively nonelastic tube. This tube, which has become lined and infiltrated by the cells of the host, must then permanently continue to bridge the blood-vessel gap to be successful. If the capacity for embryonic transplant to survive is realized, the clinical discovery and the surgery of reimplantation and replacement will certainly be as great a boon to mankind as are antibiotics.

Dr. Leighton: Dr. Greene has presented some examples of the concepts that have emerged through the study of human cancer with heterologous animal transplantation. I will devote my discussion to 2 aspects of the relationship of tumor and host and relate these to some observations and impressions gained with tissue-culture techniques.

I am particularly concerned with the stromal and vascular union between tumor cells and host tissues. As a pathologist interested in studying these problems with human tissues, I have tried to use tissue-culture methods that permit the best possible correlation between the events observed *in vitro* and those seen in tissues taken directly from the body. A combined matrix of plasma clot and cellulose sponge has been suitable for this purpose. When histological sections of such three-dimensional cultures are examined, the appearance of the cultures can often be related to the frame of reference of general pathology. The growth of connective tissue *in vitro*, including cells, reticulum, collagen, ground substance, and even capillaries, has been described by many observers using several techniques. We have made similar observations incident to studies on the mechanisms of tumor invasion. We have seen the growth of connective tissue containing capillary structures from a variety of tissues; for example, rat abdominal muscle, mouse embryonic liver, chick embryonic liver, kidney, lung, and heart, and adult human heart and synovial tissue. I would like to illustrate some aspects of this growth and relate it to studies on the stromal responses to cancer.

The outgrowths from adult human heart and synovial tissue can serve as illustrations. In a period of 4 weeks, either tissue gives rise to a luxuriant growth of connective tissue. In figure 1, plate 65, the capillary outgrowth from adult human heart stained with the periodic acid-Schiff reaction is seen. The original tissue was obtained in the operating room during an operation for a heart-valve defect, and came from the auricular appendage. The new capillaries form a branching latticework and the pericapillary sheath stains prominently. With higher magnification and, preferably, an iron hematoxylin stain, the branching structures would be seen in greater detail.

Adult human synovial tissue gives rise to a luxuriant growth of densely cellular connective tissue in which fibers, capillaries, and even intensely metachromatic ground substance are found. The capillary outgrowth from explants of synovial tissue is definite but appears to be more delicate and often less distinct than in the case of the heart cultures. In 1 small series, luxuriant cultures of synovial tissue were divided into 2 subcultures, and 1 of each pair was fed a medium supplemented with 100 γ per ml. of hydrocortisone (Solucortef®) for 16 days. On examination of serial histologic sections, the treated subcultures appeared to differ from the control subcultures in 3 qualities. The total growth was less, metachromasia was less, and the morphology of the capillaries was distinctly different. This last quality was the most definite difference of the 3. The capillaries in the cultures that received hydrocortisone were more prominent, had thicker walls, and were greater in diameter. This can be seen by comparing a field from a treated culture (fig. 3, plate 65) with an area of comparable cell density (fig. 2, plate 65).

The invasion of a host culture of synovial connective tissue by cancer cells is illustrated in figures 4 and 5, plate 65. To the right of the field was the original explant of synovia, and to the left was the inoculum of HeLa cells. The latter were added after

PLATE 65

Microscopic Sections of Human Tissues in Sponge-Matrix Tissue Culture

FIGURE 1.—A 25-day-old culture of adult heart. A narrow zone of new growth containing many branching capillaries is seen in the center of the field. Periodic acid-Schiff stain. × 60

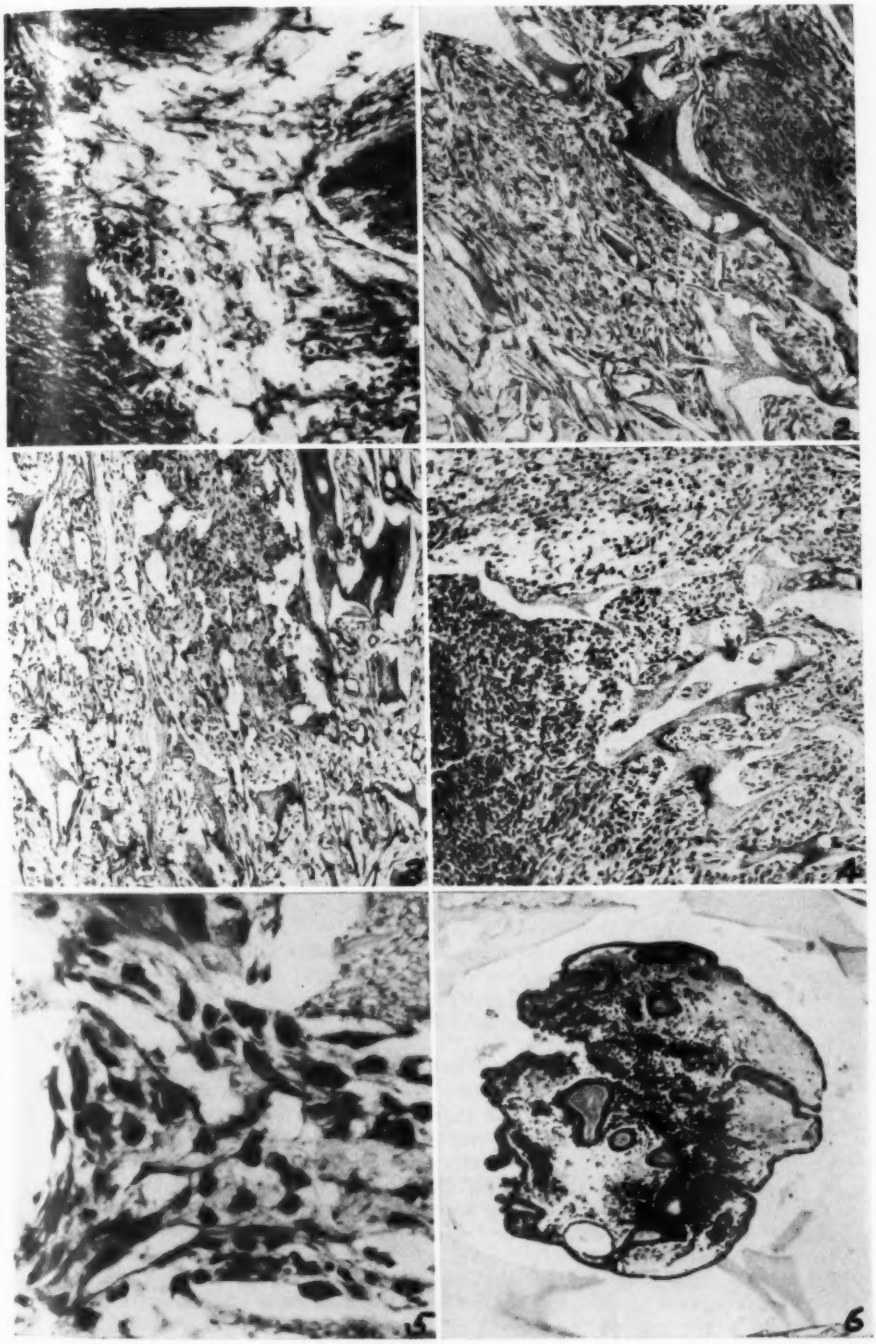
FIGURE 2.—A 40-day-old culture of adult synovial tissue. Dense connective-tissue outgrowth as illustrated in the figure occupies many of the sponge interstices. Narrow endothelial-lined structures can be identified with some difficulty at this low magnification. Hematoxylin and eosin. × 60

FIGURE 3.—A 40-day-old culture of synovial tissue treated with hydrocortisone for the last 16 days of cultivation. The hormone was added to the medium in a concentration of 100 γ per ml. of medium. Even at low magnification, widely dilated endothelial-lined channels are easily identified, as contrasted with the control seen in figure 2. Hematoxylin and eosin. × 60

FIGURE 4.—A 40-day-old culture of synovial tissue to which a small inoculum of HeLa cells was added 16 days before fixation. The HeLa cells, implanted to the left of the field, are seen infiltrating the outgrowth of synovial connective tissue. Hematoxylin and eosin. × 60

FIGURE 5.—A detail from figure 4. A narrow capillary-like channel lined by endothelial cells is seen. One of the endothelial cells lining the channel is indented by a HeLa cell. However, extension of HeLa cells into the lumens of the capillary-like structures is not found. Hematoxylin and eosin. × 240

FIGURE 6.—A 17-day-old culture of adult endometrium. Most of the surface of the explant is covered by a columnar epithelium, producing an "endometrial organism." The epithelial covering appears to have been derived by migration and growth of the glandular epithelium rather than arising from an alteration in stromal cells. Viable glandular structures and part of the stroma are seen within the explant. Hematoxylin and eosin. × 60



the growth of connective tissue was luxuriant. A detail in which a HeLa cell appears to indent an endothelial cell is seen with high magnification in figure 5, plate 65. The tumor cell has not broken into the lumen of the capillary-like channel.

In summary, the following can be stated. Capillaries grow out of a variety of tissues in sponge-matrix culture. Preliminary studies suggest very strongly that capillary morphology can be altered *in vitro* by hydrocortisone. The interaction of capillaries and infiltrating tumor cells can be studied in great detail in some combinations.

The second point concerns the desirability of maintaining human tumors in the dependent phase in tissue culture. This would be an interesting achievement, for we could then study the evolutionary changes involved in the development of autonomy. It seems to me that the growth of primary explants of cancer *in vitro* takes 1 of 2 paths. In most instances, whether in the dependent or autonomous stage clinically, a tumor grows for a few weeks and then ceases to grow. When one examines histologic sections of cultures after the period of growth has passed, the elements one finds in overwhelming abundance are those of the mature tissue; for example, a squamous carcinoma of the lung will consist of masses of keratin, and a malignant melanoma will consist of spindle-shaped cells and macrophages laden with pigment.

The alternate path leads to the establishment of a new cell line. In rare instances a rapidly growing cell line emerges in a matter of a few weeks. In many cases the cell line does not emerge until many weeks or months have passed. After a culture has looked poorly for weeks and one might be inclined to discard it, patience is rewarded by the appearance of rapidly growing plaques of cells, which, however, usually bear little morphologic resemblance to the appearance of the original tumor.

I think the cell lines that now abound will not be very useful in the study of the transition from dependence to autonomy. Methods of cultivation must be found in which the morphologic identity of the original tumor is retained in serial subculture generations. We have thought that perhaps the dependent tumor, and even the autonomous tumor, is a kind of obligate cellular parasite, which requires direct contact with normal tissue for growth. Studies are now in progress exploring this possibility. Several tumors have been combined with a variety of connective tissues. We have not seen tumor outgrowth that retains its morphologic identity indefinitely. In several instances, however, the tumor cells lived much longer and looked like the original tumor for a much longer period of time when they were grown in contact with connective tissue than when they were grown alone.

We can anticipate, in the next few years, that some of the components of the host-tumor relationship in man, which we can barely approach now with *in vivo* studies, will be examined in detail with tissue-culture techniques. Our own experience, as well as that of others, indicates that some of the mechanisms by which cancer spreads and destroys normal tissues are now accessible for intensive analysis.

Dr. Jones: The field of enzymology has been enriched in the past few years by the development of micro and ultramicro methods of chemical determinations, which can yield valuable results from tissue available in culture, from biopsy, or from pathological specimens. It is conceivable that differences, qualitative and/or quantitative, may be found in enzyme constitution, which are perhaps related to changes in tumor transplantability. In our laboratory, it has been demonstrated that the level of cholinesterase falls in chick embryonic intestine and in lung on cultivation. This level can be raised by the addition of acetylcholine to the medium Characterization of the enzyme indicates certain differences from the classic specific and the classic nonspecific cholinesterase. We have also observed that chick brain *in ovo* exposed to small amounts of acetylcholine rapidly increases in its cholinesterase activity. The role of enzyme inhibitors is being studied in these systems. Cannot changes in enzyme or in substrate influence profoundly the usual functional activity of tissue both *in vivo* and *in vitro*? Many reactions of pharmacologic importance may be examined by these methods. Perhaps a series of studies on the same tissue may reveal very important differences from the time when the fresh tissue is examined, and then at weekly intervals throughout a period of rather extended cultivation?

In the field of chemotherapy, perhaps we can now get ideas as to the mechanism of action in certain instances. For example, it has been shown that tissues from cortisone-treated chick embryos showed a prolonged inhibition of the synthesis of collagen as compared to the rate in normal untreated tissue. In addition, it was demonstrated that the presence of cortisone did not alter the incorporation of labeled sulfate into the tissue.

Chemical methods are now available for the determination of protein, lactic acid, nucleic acid, sugars, etc., in the amounts present in tissue fragments. In our laboratory, freshly explanted and cultivated tissues have been examined and comparisons made in substances isolated from the tissues and the medium. I believe that a correlation of morphological and chemical studies provides a fertile field for investigation. The very, very sensitive and excellent techniques for assay, developed by Lowry and his associates, for the right variety of enzymes can now be applied to a single cell, and, certainly now we have good chances for serial studies without destroying the whole explant or culture every time we wish to carry out a specific chemical examination. I strongly suggest that chemistry and biochemistry, although not too lateral, may be at the very center of some of our problems.

Dr. Harris: In considering these excellent experiments, which Dr. Greene has described for us, I am particularly impressed with the gulf that seems to exist between the interpretations of this body of work and that of certain other laboratories, for example, the work of Little, Snell, and others at Bar Harbor. These investigators have stressed the role of genetic factors as determinate in the transplantation not only of tumors between inbred strains of mice but also of normal tissues as well. Medawar, Billingham, and their collaborators in Great Britain have similarly stressed the role of immune mechanisms as a means of explaining the sequence of events in transplantation. Are we concerned here, in part, with a problem of semantics such as a difference in concepts of what the word "transplantability" means? Or do you feel, Dr. Greene, that there are major factors of difference in these respective bodies of work?

Dr. Greene: I feel that there are major differences. The influence of the Bar Harbor-Bethesda axis on transplantation has been very great. One must remember, however, that what they study is actually the genetics of the subcutaneous space, and it should not be forgotten that the body extends beyond the subcutaneous space to include such areas as the eye and the brain whose specialized structure greatly facilitates transplantation. Certainly the findings that apply to the subcutaneous space in Bar Harbor do not apply to the brain and the eye in New Haven. Perhaps it is merely a difference between Bar Harbor and New Haven. As far as immunity studies are concerned, I would be a little bit hesitant, because no antibody has ever been demonstrated.

Dr. Lasnitzki: In our laboratory we have implanted explants of human fetal lung, after growth in culture for 3 weeks, into the subcutaneous space of X-rayed and cortisonized rats. Eighty percent of the total number of implants were in excellent condition and grew well. They showed well-differentiated bronchioli lined with columnar ciliated epithelium and formation of cartilage. This method may be suitable for following carcinogenesis in human tissues treated in culture with carcinogens.

Dr. Klein: The laws of transplantation genetics, which Dr. Greene has referred to as findings of the Bar Harbor-Bethesda axis and as concerning the subcutaneous space only, certainly hold true in many other geographical places too, and they hold true not only in the subcutaneous space but also in the peritoneal cavity and other tissue sites as well. They don't seem to hold true in the brain or in the anterior chamber of the eye. I think it is these latter tissue sites that are exceptional, as they protect the implanted cells from the homo- or heterograft reaction of the host. The genetic laws of homotransplantation have very great merits and are by no means weakened by deviating findings in exceptional situations. Since the advent of isogenic resistant lines developed by Snell, transplantability or nontransplantability can be pinned down to a single gene difference between tumor cells and host. In fact, the host can selectively favor the survival of a few genetically compatible cells in a large incompatible population of tumor cells, even if the difference between compatible and incompatible

cells is due to a single gene. The statement that no antibody has been demonstrated in such systems is incorrect (*cf.* Gorer, P.: Advances Cancer Res. 4: 145–186, 1956). There is plenty of evidence bearing on the fact that the homograft reaction is an immunological reaction although, as Dr. Algire and collaborators have shown, in the majority of cases it is probably mediated by host cells rather than humoral factors.

Some of the discrepancy may be purely semantic. By transplantability, geneticists usually mean the ability of a tumor to grow progressively, after transplantation, and to kill the host. I am not sure that Dr. Greene means the same, by transplantability. I assume, and, if I am wrong, I am sure he will correct me on this, that he means the ability of a tumor to take and to grow temporarily in the eye or the brain, no matter whether it continues to grow progressively and kills the host or whether it ultimately regresses. When we are talking about transplantability in mice, in the subcutaneous space or the peritoneal cavity, we always mean the ability of the tumor to grow progressively and kill the animal. If Dr. Greene refers to the ability to grow for some time in the anterior chamber of the eye and without regard to the ultimate fate of the transplants, then there is a difference in semantics, which may explain a great deal of the discrepancy.

Dr. Greene: There is no question that there is a difference. I think the ability of a tumor to kill the animal depends on the site of transplantation and the speed of growth. If one transplants the V2 rabbit carcinoma subcutaneously in a DBA mouse, the tumor grows to such a large size that the mouse is unable to crawl over it to get to food and it dies of starvation. In like manner, if one transplants a human cancer to the brain of a guinea pig, the increased cerebral pressure kills the guinea pig. I don't think that eventual death is a proper criterion for the assessment of takes. Continued progressive growth, not necessarily to the point of death, is the criterion for transplantability used in our laboratory.

Dr. Wolff: I was struck by the similarity between Dr. Greene's results, with transplantations of tumors, and our own experiments on heterogeneous parabioses of embryonic organs *in vitro*. It is possible to associate *in vitro* pieces of the mouse sarcoma S180 with organs from the chick embryo. That demonstrates the similarity between both the methods of explantation and the ability of tumors to be transferred into foreign environments. In cultures, as well as in whole organisms, cancer cells show an ability to invade tissues and to produce metastases. Figure 1, plate 66,

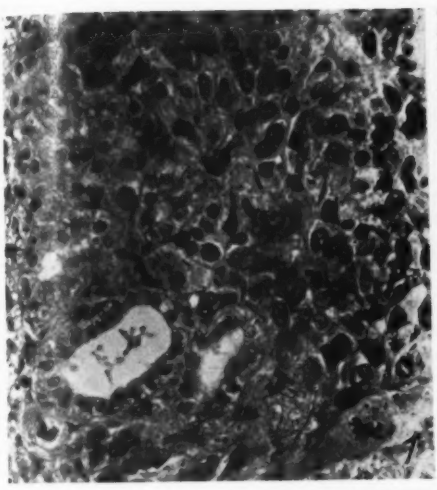
PLATE 66

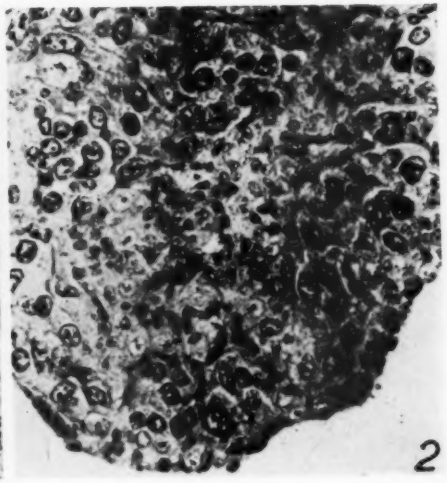
Associations between a mouse sarcoma (S180) and different chick embryonic organs cultivated *in vitro*.

FIGURE 1.—Mesonephros invaded by the sarcoma cells, which surround the urinary tubules. × 320

FIGURE 2.—Association of a chick ovary and the same mouse sarcoma. The tumoral cells are intermingled with the ovogoniae. × 320

FIGURE 3.—Invasion of the ventral skin by the mouse sarcoma. × 200

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shows a parabiosis between chick mesonephros and mouse sarcoma. The mesonephros is partly invaded by the sarcoma cells, which appear as very dark cells on the section, the lighter ones being cells of the chick mesonephros. Figure 2, plate 66, shows an association of a chick ovary and the same mouse sarcoma. The cells of the 2 species are absolutely intermingled; the dark tumor cells invade the cortex and take the place of the ovogoniae. It appears that there is no incompatibility between the 2 kinds of cells, especially in the gonads. Figure 3, plate 66, shows an association between the cancer cells and the somatopleura of a 10-day-old chick embryo. The larger tumor cells are seen invading the connective tissue of the chick, which is still surrounded by the epidermis. It is very striking that in embryos, as well as in the examples referred to by Dr. Greene, transplantations or parabioses produce similar results.

Dr. Leighton: Dr. Wolff, as you follow combinations of tissues, do the normal tissues diminish progressively in cell population and size as the tumor cells grow, or is an equilibrium achieved in which there is no further decrease of the normal tissue?

Dr. Wolff: In the beginning the normal cells continue to grow, but the cancer cells very rapidly invade all the organs and replace the other cells. It is not an association, but a true parasitism.

Dr. Leighton: We made a similar observation in combinations of HeLa cells with connective tissues of the chick embryo. The embryonic tissues were progressively replaced and, finally, HeLa cells were the only living cells left.

Dr. Greene: I would like to ask Dr. Leighton a question. Were the capillaries that you observed, which appeared to be patent, functional?

Dr. Leighton: We have a few scanty observations suggesting that the capillaries can communicate with the medium, at least in some instances. Cultures of chick embryonic heart make a prominent capillary bed. In 1 experiment, a culture of chick embryonic heart was combined with bloody fluid from the pleural cavity of a patient with carcinoma. Although the tumor cells failed to grow for any length of time, sections of the combination showed many of the newly formed capillaries of the chick embryonic heart filled with red cells. I think this indicates that some capillaries are patent; it even suggests that they may be in communication with the medium.

Dr. Harris: Dr. Wolff's remarks impel me to ask Dr. Moscona a question with reference to the paper on the aggregation of dissociated cells. Dr. Moscona, have you ever used dissociated tumor cells and normal cells to see whether they aggregate separately, as you have shown with different tissue types in your experiments, or whether they would intermingle as is apparently the case in the explants described by Dr. Wolff?

Dr. Moscona: We have made observations on several combinations of tumor cells and normal, embryonic cells. They may soon be ready for summary. Confronted with dissociated tumor cells, discrete embryonic cells behave, as far as we have seen, type-specifically, that is, following aggregation, they form groupings, the distribution of which within the aggregates depends on the types of cells involved. Hepatic cells, intermingled with S91 melanoma cells, tend to group on the surface of the cluster. Cartilage cells aggregated with S91 cells are consistently found inside the aggregate. Eventually, however, following the establishment of coherent tissues, the tumor cells begin to invade the normal tissue. Here may be a point of contact with Dr. Wolff's observations.

Dr. Greene: Embryonic tissue will grow anywhere in the body of a heterologous host. It doesn't have to be transferred to the eye or brain as cancer does.

Dr. Wilde: I would like to point out another tool in the study of relationships between host cells and malignant cells. This is seen in the Breedis experiments. (Breedis, C.: Effect of temperature on a neoplasm-regenerate complex in the newt, *Triturus viridescens*. (Abstract.) Fed. Proc. 13: 1954). There is an induced newt sarcoma that remarkably resembles the amphibian regeneration blastema. If an implant of the sarcoma is made into a limb, and after the graft has taken, the limb is amputated, and the normal regeneration of the host will proceed from the normal

tissue. One would expect some sort of response from the sarcoma tissue that has also been transected by the guillotine operation. Breedis has found that tumor response is remarkably temperature sensitive. At low temperatures, the tumor keeps pace with and grows side by side with the regeneration blastema that differentiates into cartilaginous phalanges, muscles, fingers, etc., while the tumor behaves in a very tame manner. On the other hand, at higher temperatures, the tumor rapidly overtakes and swamps out the activity of the regeneration blastema. If you have aggregates of malignant and nonmalignant cells, temperature control *in vitro* or *in vivo* may prove to be an exciting tool.

Dr. Algire: Dr. Greene made grafts from homologous or heterologous tissue into animals that had received prior inoculations of such homologous or heterologous tissues. Did they take?

Dr. Greene: Yes, human cancer transplanted to guinea pigs immunized against human tissues grows as well as it does in normal pigs.

Dr. deBruyn: I have a malignant strain of lymphoblasts growing together with fibroblasts in the same culture, a mixed culture. The lymphoblasts grow together not only with fibroblasts derived from a mouse of the same strain in which the tumor developed spontaneously but also with fibroblasts derived from a mouse of another strain. It is even possible to cultivate them together with fibroblasts of the rat.

However, to let the lymphoblasts grow and produce the tumor *in vivo*, it is absolutely necessary to inject them into a mouse of the same strain in which this tumor developed spontaneously. So there is a difference in behavior of the malignant lymphoblasts *in vitro* and *in vivo*.

Dr. Greene: I have never been able to transfer a human lymphosarcoma, Hodgkin's disease, or a tumor of the myeloid tissue, heterologously, which suggests that they are unlike the ordinary cancers. Perhaps they never become autonomous. I should qualify that with reference to one animal tumor. Very recently, I have succeeded in transferring the Gardner lymphosarcoma of mice to the subcutaneous space of hamsters. The incidence of takes in the first generation was 1 in 30, and this has not increased with continued passage.

Dr. Willmer: Can some of these cancers that have been successfully grafted into the eye and have "taken" then be transplanted into the skin and kept growing?

Dr. Greene: I have never used the subcutaneous space for such grafts, but you can transfer second-generation growth of human cancer to the testicle or musculature of other animals.

Dr. Klein: Do they regress later?

Dr. Greene: Yes, unless they are located at a site where their continued growth impedes a vital process. I have seen several lymphatic extensions from transplants but no metastasis.

Dr. Gey: We have had some interesting observations, which someone here may explain. When our HeLa line was well established in culture for over a year, it was transplanted into the anterior chambers of a group of guinea pigs, and 1 guinea pig showed an interesting progressive growth of the tumor on the iris. This resulted in a beautiful palisading and organization of the tumor cells on the borrowed basement membrane of the iris stroma. In the same eye, there was good growth on the lens capsule but not especially differentiated. In the retina there was marked invasion and destruction. Subsequent efforts to transplant our strains of HeLa into the guinea-pig eye have failed completely even when the guinea pigs were treated with estrogen as in the more successful original transplants. We have been unable to account completely for this failure to get regular transplantation of HeLa in the guinea pig's eye. Yet others have transplanted it heterologously following cortisone and X ray in rats and hamsters and, homologously, in tumor-bearing patients. Some of these changes in the behavior of cultured tumor cells need a more careful evaluation.

Dr. Greene: I have had exactly the same experience. I tried to transplant some of Dr. Leighton's sponge preparations of growing human cancer in pigs' eyes and failed completely.

Dr. White: Dr. Green usually has in his pocket 1 slide, which he has failed to show this time. A number of years ago, when we were associated at Princeton, he took some of my plant cultures and put them in the eye of the rabbit. They certainly enlarged very markedly. The sections look very much like some of the things we see here. You can feed carrot to the rabbit by several different channels.

Dr. Greene: I have tried since then, Dr. White, to grow cabbage in cow's eyes so that we could have corned beef and cabbage on the hoof.

Dr. Gaillard: There are two points I want to make. First, in relation to the possibility of having cells of different origin together in tissue culture, I should like to report that human cervical carcinoma can be grafted into an explant of a fragment of rat intestine. If you make a rat intestinal-organ culture, the intestinal epithelium will grow and surround the explant and within this intestinal sac you will have a mixture of connective tissue and smooth muscle tissue. Now, pinching a little hole in that explant and introducing a fragment of cervical carcinoma in it, the two types of tissue will, at least in short-term experiments, stay together. The tumor starts infiltrating the connective and muscle tissues from the inside toward the periphery.

My second point is that as far as grafting procedures are concerned, I do not think that it is fair to doubt that there are antibody responses in the organisms. On the contrary, we will have to explain that homografting and, perhaps, even heterografting in the brain and in the anterior chamber of the eye obviously *are* possible. Now also in the human being, we have done some grafting with embryonic human tissue. We have, as far as this is concerned, two examples. One is a series of grafts of cultivated fetal human parathyroid gland in the perivascular lymph spaces of the brachial artery of patients suffering from a postoperative tetany. We started this work in June, 1941, and up to date, we have 12 positive takes and we were lucky that our first take was positive. This extremely well-controlled patient is without any appreciable symptom since approximately October, 1941. Quite recently, but perhaps too soon to report, though I think it is worth mentioning in connection with what has been said here, we have tried to graft human fetal ovarian tissues, grown *in vitro*, into patients suffering from an ovarian agenesis. In 22 or 23 cases we got completely negative results until we played a little trick. This is to do a two-step operation. During the first laparotomy, we took from the future recipient a little fragment of peritoneum about 1 cm. square and brought it on top of a clot for 1 day only. On top of this "big" explant that is on the peritoneal side, about 100 of those tiny ovarian cultures were placed. The two types of tissue grow together in the course of 24 hours, and then the fragment of peritoneum with the little fragments attached to it was placed into the peritoneal wall of the patient. In the case of an ovarian agenesis, there is a very nice possibility of controlling the possible "take" of the graft because one can estimate the follicle stimulating hormone (FSH) content in the blood. The FSH level in patients suffering from ovarian agenesis, as a rule, is extremely high. During the first 2 months, after grafting in both patients treated in this way, the FSH levels went down and down. It is now approximately 1½ years ago, and the FSH values have remained approximately normal. This finding indicates that the grafts must have produced estrogenic substances that were responsible for inhibiting the FSH production of the anterior pituitary.

Dr. Moscona: Dr. Greene, in your heterologous transplant did you notice whether the connective tissue of this graft was that of the host or from the implant? Do you think this connective tissue has anything to do in these cases of remarkable acceptance of the graft?

Dr. Greene: One of the first questions that arose following the successful transplantation of a human cancer to a pig's eye concerned the species identity of the transplant. Did it remain human or did it become guinea pig? It was found by means of complement-fixation reactions utilizing a variety of different cancers that irrespective of the donor species, the growing transplant manifested only the antigenic properties of the species in which it was resident at the time of the test. Inasmuch as the connective-tissue content of the tumor changed to that of the new species following transfer and the species specificity of the transplant varied accordingly,

such reactions could be attributed to its stromal component. However, the parenchyma of the transplant is not a product of the new host but persists on transfer, and its failure to react with any of the antisera of the original host species is a significant finding.

In another series of experiments, the saline-soluble cytoplasmic components of heterotransplants were separated and their species characteristics studied by rigidly standardized procedures. It was found that they had undergone a complete alteration and, as in the previous experiments, appeared to be under the direct influence of the host supporting the growth of the transplant. Such an influence was confirmed by returning the tumor to the species of origin where it was found to possess again the cytoplasmic components characteristic of that species.

The mechanism whereby the cancer cell attains the unique property of responding to the influence of the foreign host by altering the chemical constitution of its cytoplasmic components to correspond with that of the new species is the subject of continued investigation.

Dr. Jacoby: I think these results with heterotransplants of tumors are very remarkable. I should like to ask Dr. Greene whether he could tell us a little more about the morphological types of tumors involved; whether they belonged to so-called precancerous types of growth or to tumor types the malignancy of which is often very difficult to diagnose from histological specimens and for which prognosis has been evolved as it were merely by agreement among pathologists. And whether, by this transplantation method, one might have in hand a sort of biological test for prognosis of tumors that are histologically notoriously difficult to assess with regard to malignancy?

Dr. Greene: There is no morphological difference whatsoever between heterologous transplantable tumors and those that fail to grow on transfer. They were all cancers under the microscope. This has led to great difficulty for me because I am a surgical pathologist. It is necessary for me to substitute the "prognosis" for "diagnosis" on the surgical pathology diagnosis sheets. Thus, instead of a diagnosis of cancer I make a prognosis which means that if the tumor remains in the patient long enough, it will develop the ability to invade and metastasize, but I can't tell by looking at the tumor under the microscope whether or not it yet has such abilities. There is no morphological difference, that I can detect under the microscope.

Dr. Klein: Just a word with regard to stroma and tumor cells. If you take an ascites tumor of an inbred mouse strain such as strain A and inoculate it subcutaneously into an F_1 hybrid between strain B and A, it will grow in 100 percent as a solid tumor. This tumor has an A $\times$ B stroma. But it still behaves as an A tumor and will continue to do so no matter how long it is carried in the hybrid. A $\times$ B tumors are not transplantable back to A mice. Tumors that originated in A but are carried in F_1 always are transplantable. This and a series of other examples can be quoted to support the idea that the homograft reaction, at least in the mouse, is primarily a reaction against the tumor cells and not against their stroma.

The Future of Virus Studies in Tissue Culture [1,2]

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The subject of this paper, for which I immediately disclaim responsibility, inevitably casts me in the role of prophet. I have assumed this role with some misgiving. First, because I am impressed with the truth of the Biblical maxim concerning the unfavorable light in which his colleagues are apt to regard the prophet; secondly, because the history of science reveals how often quite unexpected discoveries or inventions of new techniques have directed the stream of advancing knowledge into channels that were quite unforeseen. In this instance, however, I agreed to act in the capacity of a seer because there can be little doubt that tissue culture will afford one of the most effective means whereby our knowledge of viruses will be further increased. Moreover, the beginnings of the investigative pathways, almost certainly to be broadened and extended with the help of this technique, are already so clearly established that one does not run great risks if he attempts to envision some of the directions they are now likely to take.

These pathways are, of course, numbered by all the fundamental and practical problems in the field of virus research. Lack of time, as well as incompetency, compels me to omit direct consideration of the majority. Among these omissions must be the applications of tissue culture to such basic matters as the analysis of the physical and chemical properties of viruses. Neglected also will be the important part that tissue culture will doubtless have in the further eludication of other such fascinating phenomena as recombination of elements derived from different viruses, and the capacity of one virus to interfere with the multiplication of another. I also regret that I shall not have the opportunity to consider how certain practical applications of the method will be extended and improved. One can confidently expect, however, that it will be used effectively in the future, as in the recent past, to isolate new viruses and to establish their relationship to the disease with which they are associated. Also, in the diagnosis and prophylaxis of known viral diseases and in the study of epidemics in which, within the last few years, tissue culture has

[1] Received for publication May 28, 1957.
[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8-12, 1956.

assumed a position comparable to that of the classical methods employed in the cultivation of bacteria, it will be constantly invoked as an essential procedure.

I am forced to limit my comment to two of these many areas of investigation. The first encompasses those factors upon which the natural susceptibility or resistance of the cell to infection by virus depends. The second, and it is closely related to the first, includes the mechanisms that underlie viral cytopathogenicity. Even when confined to these topics, the discussion must obviously be exceedingly fragmentary. The illustrative material to be presented will be drawn from studies on mammalian viruses and their host cells, because I am familiar only with such systems. To what extent certain of the phenomena that I shall mention will find or have already found their counterparts in other host-virus systems has not yet been clearly determined. Indications, however, are increasing, as research is intensified on viruses attacking widely different hosts, that they are similar in respect to many of their basic properties and activities. So it is possible, as Maramorosch among others, suggests, that "The still prevailing and very convenient division of viruses into bacteriophages, animal, plant, and insect viruses, may have to be abandoned in the future; like bacteria which are no longer divided according to the hosts they attack, viruses will come to constitute a unified field of study."

It has long been recognized that just as different species differ in their resistance to infection by a given agent, so different kinds of cells within the same species may vary in their reaction to the presence of virus. The recognition of differences in cell susceptibility within the animal body formed the basis of an early classification of mammalian viruses according to their apparent neurotropic, dermotropic, or viscerotropic properties. During the lengthy period from 1914 to about 1950, when many investigations on the growth of viruses in tissue cultures were carried out, relatively few attempts were made to determine whether, under these altered conditions, various kinds of cells would continue to exhibit the same degree of susceptibility or resistance they show within the living animal. The paucity of such attempts cannot, I think, be attributed to a failure to appreciate their importance, but in part, at least, to a lack of adequate techniques whereby cultures consisting predominantly or entirely of one cell type could be readily prepared and maintained. In the absence of accurate data, divergent views were prevalent. Thus certain workers considered that cells in culture remain essentially unchanged, while others believed that marked variations in their resistance or susceptibility might occur. The question arose as to whether changes that followed cultivation *in vitro* might render cells originally derived from completely resistant species, susceptible to infection. The demonstrations in 1949 and 1950 that poliovirus—then regarded as a strictly neurotropic virus—could be cultivated in non-nervous tissues and that the cells in which it multiplies are injured and destroyed, have, among many other recent observations, served to renew interest in these old and fundamental problems that still await solution.

Fortunately this renewed interest can now be implemented by the recent advances in the techniques of tissue culture as applied to the study of animal viruses. Owing to the insight and effort of such workers as Earle, Gey, Syverton, Scherer, Dulbecco, Puck, and their collaborators, we are prepared to restudy these phenomena with the aid of systems consisting of one cell type or even of a single clone. The value of these methods in the analysis of cell resistance will continue to be enhanced, I think, as means are found for the ready cultivation of additional types of cells which so far have offered difficulties, such as those of the liver parenchyma.

The objectives of some of the investigations, which will rely heavily on these procedures, can be easily visualized. First, there is the highly desirable objective of collecting the large body of data that would permit the formulation of a comprehensive and systematic account of natural cellular resistance to the known mammalian viruses. This account would be based on studies of the reactions of the principal cell types of man and the common domestic and experimental animals and include information respecting the resistance of the same cell types as exhibited in the living animal. It may be long before there will be sufficient material, but, eventually, an account of this nature should be possible. Moreover, when finally completed, it may well include sections not only on bacteria and phages but also on the interactions of viruses and the cells of insects and plants; for I have confidence that techniques will be developed whereby the cells of insects can be readily cultivated, and those of plants rendered susceptible to infection *in vitro*.

I doubt, however, whether the study of cellular resistance in tissue cultures will prove entirely satisfactory, or the investigation of other problems in virus-host relationships, unless the workers concerned agree to adopt measures that will promote uniformity and, therefore, comparability of results. As I see it, the most important measures would be *a*) the designation of representative or prototype strains of each species or antigenic type of virus to be used by everyone engaged in this kind of research; *b*) the maintenance of stock cultures of these agents under conditions that would tend to minimize or prevent the occurrence of variation or mutation; *c*) the selection of representative strains of cells that preferably would consist of single clones and be grown under conditions designed to reduce changes in their resistance to the prototype viruses. It might also be stipulated that when it is necessary to employ cells that have not been propagated in continuous culture, uniform procedures would be employed in the preparation and maintenance of original explants. The rich harvest of results that followed the adoption of measures similar to these by students of the bacteriophages should encourage us to imitate their example at the earliest possible moment.

It is, however, open to question whether this moment has arrived, since we do not yet have many types of normal cells in continuous culture, nor can we be certain of assuring invariability in those we have. I am hopeful, nevertheless, that the intensive work in progress on the development of new lines of cells, on their requirements for growth, and on their preser-

vation and storage will soon enable us to make common use of reasonably uniform materials.

For purposes of analysis, the phenomena of cellular resistance are naturally divisible into two main groups. To one belong the mechanisms involved in the penetration or exclusion of the virus particle; to the other, the intracellular factors upon which viral multiplication depends or by which it may be repressed.

I shall first comment on the factors that condition the passage of virus into the cell. *A priori* these seem to be fewer and less complex than those concerned with multiplication of the agent. Nevertheless, we are far from having a clear picture of even these factors except perhaps in the case of certain bacteriophage systems. For the animal viruses, the most desirable approach to future exploration of this problem appears to be through the use of tissue-culture cells. Up to this time, suspensions of erythrocytes have been extensively employed as substitutes for tissue cells. Although much interesting and valuable data have thus been accumulated, it would be preferable, I think, to base final conclusions concerning the mode of attachment and entrance of virus on direct observations of tissue cells such as those Dr. Bang showed us, since it is only in these elements that viral multiplication has been shown to take place.

If I am correct in this view, one of the first steps that should be taken is to determine whether cell susceptibility is invariably correlated with fixation of virus on the cell surface and conversely whether resistance is invariably characterized by failure to fix the virus. Comparative experiments have been rare. A recent investigation, however, by Kaplan, in Melnick's laboratory, indicates that such correlations may hold in the case of poliomyelitis virus. Much more information of this kind is needed. It could be obtained most rapidly and profitably by collaborative investigations in which standard viruses and cell suspensions are employed. A combined effort of this sort would be justified, I believe, since it might afford an unequivocal demonstration that absolute or complete resistance is dependent solely upon properties that result in exclusion of the virus at the cell surface. If so, it would obviously waste time and effort to search for the mechanisms of resistance within the more complicated internal environment of the cell. On the other hand, if it should turn out that virus attaches to the completely resistant cell and thereafter regularly penetrates within, it will be necessary to proceed to the much more difficult task of discovering whether its failure to multiply is negatively conditioned by defects in the biochemical equipment of the cell or by sustances or metabolic processes that exert a positive antiviral effect.

I shall not attempt to describe the details of the procedures that might be employed in experiments to decide the question of whether resistance depends on surface or internal factors. It will be enough to suggest that the newer techniques provide a simple means of bringing cells into contact with virus, and of removing unabsorbed virus from the system. It should then be possible, on the basis of evidence now avail-

able, to detach the virus absorbed to the cell surface by treatment with the specific antibody in the cold. Any residual virus associated with the cells should represent what has penetrated into the interior.

So far, I have, in the main, been considering the sort of cellular resistance that precludes any multiplication of the virus; that is, complete or absolute resistance, analogous to the absolute resistance of one species to a virus highly virulent for another. I should now like to refer to a kind of cell resistance, which for the purposes of this discussion we may term partial resistance, and which may be compared to the so-called immunity of infection in the living animal. Indeed, there can now be little doubt that the immunity of infection is maintained by the presence in the tissues of infected cells that exhibit incomplete resistance to a given virus. This type of cellular resistance is responsible for what has been recently referred to as the "carrier state" and is comparable to lysogenicity in bacteria. It is by means of the tissue culture that its existence was unequivocally established and it will almost certainly be by this technique that its mechanism will be finally understood.

Many years ago, with Feller and Weller, we carried out long-term experiments on the growth and multiplication of vaccinia virus in roller-tube cultures of mixed chick embryonic cells. It was observed that virus production continued at a maximum for many weeks in the absence of grossly apparent injury to the cells. In fact, cell growth occurred at about the same rate as in uninoculated cultures. While microscopic examination of stained specimens revealed an occasional cell containing a cytoplasmic inclusion body, the majority were normal in appearance. In 1952, Bang and Gey published the results of a study on the varying susceptibility of 13 established strains of rat cells to Eastern equine encephalitis virus. Included among these cells were both malignant and normal lines. A broad spectrum of variation was demonstrated ranging from complete or almost complete resistance to extreme susceptibility characterized by rapid multiplication of virus and destruction of cells. Partially resistant lines continued to support virus growth for many months and through a series of transfers. In certain instances, the virus eventually disappeared from cultures in which the cells continued to proliferate. Such variations were not dependent on the malignancy of the cell line. A most interesting example of partial resistance on the cellular level has lately been presented by Puck. HeLa carcinoma cells were shown to be susceptible to infection by the Newcastle virus disease of chickens. When these cells were maintained in the usual manner, however, the cytopathogenic effects of the virus were observed to be minimal or absent, although multiplication of the virus continued. When noninfected HeLa cells, previously irradiated with X rays, were exposed to cells carrying the virus, destructive changes in the irradiated cells promptly occurred. Here we have a new tool comparable to systems of susceptible and lysogenic bacteria for the future exploration of this exceedingly important phenomenon of partial cellular resistance to viral infections. It should be of much value in working out the details of the

responsible mechanisms, as the progress of biochemistry distinguishes the steps in the synthesis of nucleic acids and proteins and provides the means of recognizing in these substances the slight differences in molecular structure upon which, in all probability, depend the reactions of different cells to different viruses.

In concluding these random remarks on this subject, I shall yield to temptation and give another example of partial cell resistance drawn from our own recent experience and encountered during an investigation that is being carried out in collaboration with Dr. Herald Cox and his associates, of the Lederle Laboratories. In 1952 Roca-Garcia, Moyer, and Cox succeeded in adapting the MEFI strain of type II poliovirus to chick embryos. Following adaptation to this host, it was found that the virus had largely lost its original capacity to destroy monkey renal cells in tissue culture. Since it also failed to produce gross injury in the chick embryo, it was necessary to inoculate mice with material from the embryo in order to demonstrate multiplication of the virus and to assay its infectivity. For this and other reasons, it became of interest to discover whether or not the agent had also lost cytopathogenicity for certain varieties of human cells. At the request of Dr. Cox, experiments to elucidate this point have been carried out in my laboratory. We have confirmed the observation that this agent caused no definite destruction in cultures of renal epithelial cells from rhesus monkeys. Similarly, in cultures of postnatal human kidney cells, we have been unable to discern degenerative changes that were clearly attributable to the virus. Both human and monkey renal cells, however, are not completely resistant to infection since we have obtained evidence that they have supported viral multiplication over a period of about 3 months of serial passages. It is noteworthy that during this time no indication of a resumption of cytopathogenicity in respect to renal cells has been observed. Cells of the line established by Stulberger from human bone marrow and designated Detroit 6 have likewise proved partially resistant. Following infection of these cells with the egg-adapted poliovirus, cytopathogenic changes have not been recognized, yet active multiplication of the agent has been demonstrated. In sharp contrast, and this is a very striking difference, epithelial cells of the mature human amnion and the spindle cells growing from human myometrial explants are rapidly destroyed by this strain of virus, just as they are by all other polioviruses that have been examined.

In this instance a viral mutant served as an instrument to reveal striking differences in the resistance of human cells derived from various anatomical sources. Particularly interesting is the variation in the response of the 2 kinds of human epithelial cells—cells that, morphologically, closely resemble each other. There must be essential differences in metabolic processes underlying these variations in response to infection, which, if they are ever defined in chemical terms, will help materially to place immunology among the exact sciences. In the definition of these subtle differences, the biochemist will surely be aided by tissue-culture techniques.

Examples of the capacity of viruses to injure and destroy cells in tissue culture were infrequently recorded in the past by workers such as Huang, Ivanovics, and Hyde. The practical value of the phenomenon, however, was not generally appreciated until about 6 years ago. Since that time many species of mammalian viruses have been found to exhibit cytopathogenicity under appropriate conditions. This property, which has been ably pointed out this morning, is of much practical significance because it has rendered the tissue culture independent of the experimental animal as a means of detecting the presence of cytopathogenic viruses and as a measure of their infectivity. Unless someone succeeds in cultivating viruses in cell-free media, tissue culture will, in most instances, offer the method of choice for these basic operations.

Now, however, I would stress the opportunity that it provides for the future analysis of viral cytopathogenicity, for to my mind it is difficult to imagine an enterprise of greater intrinsic biological interest. I feel this way not only because I am primarily concerned with the viruses themselves, but also because I recognize that they may be used as helpful tools in the future study of the normal as well as the abnormal physiology of various kinds of cells.

That viruses can be used in this way is implicit in the rapidly increasing number of observations showing that the morphological response of cells to different species of virus as well as to variants of the same species may be strikingly diverse. Surely such differences in the appearance of infected cells must reflect injury to quite different physiological mechanisms. If this is true, it may prove feasible, in the tradition of Claude Bernard, to employ viruses with different cytopathogenic properties to produce specific intracellular physiologic lesions. The effects of these lesions could then, perhaps, be investigated by biochemical and biophysical methods. As one without any competency in these disciplines, I can merely suggest these possibilities which, of course, are in the minds of all who daily observe the remarkable transformations that cells undergo as the result of viral infections.

To emphasize the wide variation in cytopathogenic changes caused by certain viruses, I shall describe those produced by measles virus with which we have recently been concerned. When the strain we have designated "Edmonston" was first isolated in cultures of human renal-epithelium cells, alterations appeared, after a lapse of several days, as shown in figure 1, plate 67. These effects have been consistently reproduced in each of 26 serial passages of the virus in these cells. We are inclined to believe that the large syncytium-like masses or "multinuclear giant cells," which are the striking feature, result from the coalescence of the cytoplasm of a number of adjacent cells and not from nuclear divisions within a single cell. If this hypothesis proves correct, then one of the first effects of viral multiplication is either to damage the cell membrane directly, perhaps by toxic action, or indirectly, by injuring the mechanism responsible for maintaining the membrane's integrity. During a period of about 1 week after the giant cell has formed, the nuclei maintain a normal ap-

pearance. Subsequently, they are seen to contain small inclusion bodies. At about the same time large masses of densely eosinophilic material gathers in the cytoplasm. That this material is associated with the viral antigen has been demonstrated by the fluoresence antibody technique of Coons.

After 23 passages in human renal cells, the Edmonston strain was propagated in cultures of human amnion cells during 26 serial transfers. In this medium, it displayed, at first, the same cytopathogenic properties. However, after several passages in amnion cells, the number of giant cells tended to diminish. At the same time many elongated, highly refractile cells were observed that resembled fibroblasts. By the 26th passage in amnion cells, this effect was predominant.

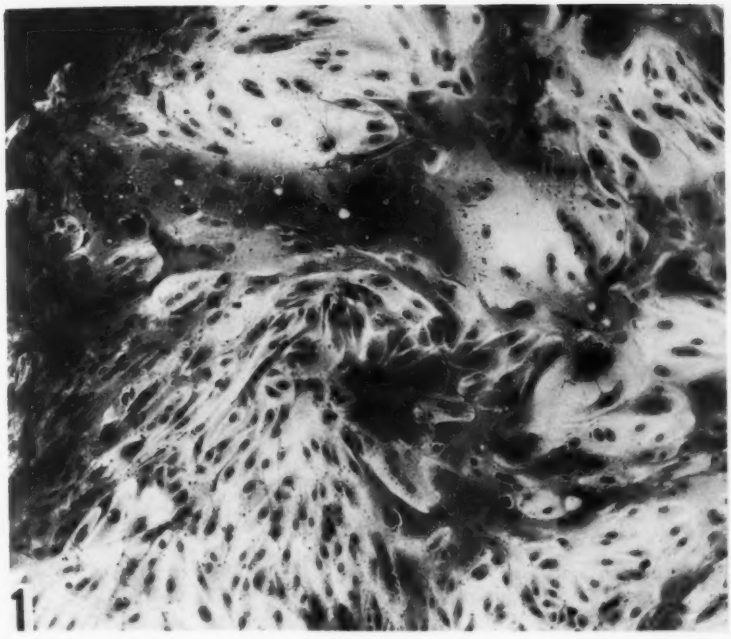
Numerous attempts to demonstrate multiplication of the Edmonston strain as well as several other strains of measles virus in chick embryos failed when the inoculum consisted of virus propagated in human renal cells. The variation in cytopathogenic properties of the Edmonston strain that occurred during the amnion cell passages suggested the possibility that the virus might be more easily adapted to growth in the chick embryo. Acting on this thought, 5 serial passages originating with the virus of the 26th human amnion cell passage have so far been carried out in this host. Calculation of the dilution factor involved in these transfers leaves no doubt that multiplication has occurred. The identity of the virus present in the fourth egg passage has been confirmed by the results of neutralization tests. Its cytopathogenic properties, as previously exhibited in cultures of human amnion cells, have remained unaltered during the egg passages. The changes produced in cultures of amnion cells by the egg-passaged virus are illustrated in figure 2, plate 67.

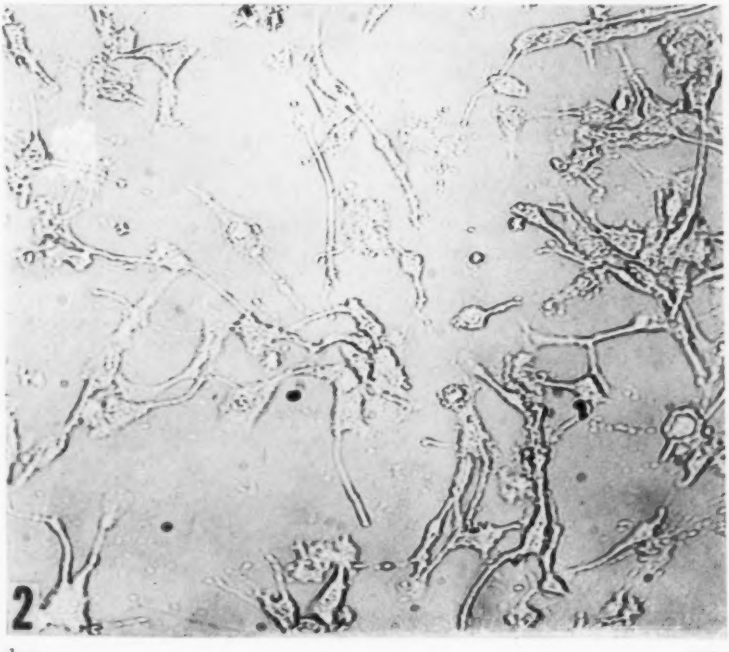
I realize that I should not have donned the prophet's robe, since I only commented on things whose outlines are presently so clear that no prophet is needed to trace the shapes of the shadows that they cast before. As an excuse, I can only urge my enthusiasm for the method of tissue culture, and my firm faith in its rich future.

PLATE 67

FIGURE 1.—Edmonston measles virus—25th passage in cultures of human renal cells. Appearance of cells 16 days after addition of the virus. *Note* syncytia or giant-cell formation. Bouin's fixative. Hematoxylin and eosin. × 105

FIGURE 2.—Edmonston measles virus. Effect of virus on human amnion cells in tissue culture after 3 passages in chick embryos. Appearance of cells 14 days after addition of the virus. *Note* spindle-cell forms. Unstained. × 180





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DISCUSSION

Dr. Black: I should like to invoke the protection of Dr. Enders' introductory remarks because I feel they apply much more to me than they do to him. Very rarely am I willing to admit, particularly in public, that some plant-virus field is lagging behind an animal-virus field. However, in this particular case, the situation is so obvious that perhaps the best service I may perform is to restate the nature of the problem with which plant virologists are confronted. I hope, of course, that plant virologists may be able, in the future, to use the wonderful techniques that were first developed with bacteriophages and recently applied to animal viruses with such success. I would like to congratulate the animal virologists on their tremendous activity in the culturing of discrete animal cells and the culturing of sheets of animal cells showing the interactions between single virus particles and single cells and in plaque formation in sheets of cells. I am also optimistic about the future; I feel sure the major obstacles confronting the plant-virus fields will be overcome.

What are those obstacles? The plant cell is surrounded by a cellulose wall and, in nature, apparently all plant viruses are introduced into this cell by means of an extrinsic agent, usually by the sucking mouth parts of an insect. How do the plant viruses get out of the infected cell? Again, the release is almost always engineered by means of an extrinsic agent. In the intact plant, viruses presumably spread from cell to cell through protoplasmic connections that penetrate through the cell walls. In the cases tested thus far, inoculations of single plant cells in culture have resulted only in failures. I admit that, at the present time, only preliminary experiments have been made.

I do not want to give the impression that a plant virus represents a simple, unspecialized, unadapted inert mass of nucleoprotein. We know that this is not true. The plant viruses show a remarkable specificity in their transmission by different groups of insects. A virus transmitted by leafhoppers is not transmitted by such insects as white flies, aphids, mealy bugs, etc. Conversely, a plant virus transmitted by white flies is not transmitted by any of the other insect groups. Although the viruses may look very similar under the electron microscope, nevertheless there are group specificities or even more refined specificities that are determined by the heredity of the particular plant virus. Another indication that the seeming simplicity of the individual viruses is illusory is the fact that these viruses may lose their transmissibility by vectors. This change is hereditary, but the changed virus may appear just the same under the electron microscope as a virus that can be transmitted.

It is probably idle for me to speculate on the possible future course that will result in surmounting or circumventing the difficulties of plant virologists in using cultures of discrete cells or cultures of sheets of cells as the phage and animal-virus workers do. It is more likely that any particular speculation will be sterile rather than fruitful. But there are one or two ideas that may be worth mentioning. One encouraging aspect of the animal work in tissue culture in relation to virus is the expansion of the host range of the viruses. A particular virus sometimes becomes able to attack more than the specialized type of cell that it may attack *in vivo*. And a particular virus is often capable of attacking cells of a greater number of species. There is a possibility that even some of the plant viruses could be grown in animal cells. If this could be done they might behave like animal viruses. There is some indication of this in those plant viruses that actually multiply in their insect carriers. The evidence indicates that these viruses are able to penetrate into the tissues, multiply in those tissues, and be released from them. In other words, when they are in the insect, they behave like an animal virus. There is also the possibility that, if there were a sufficiently broad

base of work on the production of the various plant cells in tissue culture as single cells, by treatment or by mutation or by some other method, plant cells might be obtained that would be free of the cell wall. Plant cells free of their walls would seem to offer a better chance of success for use of the phage techniques with plant viruses. In any work of this sort, it should be remembered, of course, that plant viruses, in general, are active at about 10° C. lower than are the viruses of warm-blooded animals.

I have sounded, what must seem to many, a rather gloomy note on this particular field of plant virus investigation, and I hope there will be an opportunity later for Dr. Kehr to show a few slides that illustrate one more hopeful aspect of plant virus investigation in relation to tissue culture.

Dr. Hotchin: Future virus tissue culture research is likely to travel along 2 divergent paths. On the one hand, the investigation of the role of viruses in the causation of disease will undoubtedly progress; on the other hand, I would like to suggest that the use of virus as a tool to investigate intracellular physiology will be increasingly developed, particularly by those who are less interested in the clinical aspects of disease.

Let us briefly consider the first path; there are many unanswered questions such as the fundamental riddle of cytopathogenicity. We really know very little about the importance of this effect in disease and nothing concerning the biochemical lesion in the affected cell. Practically nothing is known of the detailed nature of the interference phenomenon, and, as yet, we are making no real use of this potentially valuable property of viruses. It would be trite to say that there are many interesting things to find out in terms of the relationship between virus growth and cellular differentiation, but it seems fair to claim that with the present technique, some of this information is within our grasp. My feeling is that the most fruitful approach, which lies before us at the moment, would result from the marriage between animal investigation and precise tissue-culture methods. In this connection I have recently become interested in lymphocytic choriomeningitis infection in mice. Traub, some years ago (Traub, E.: Epidemiology of lymphocytic choriomeningitis in a mouse stock observed for 4 years. J. Exper. Med. 69: 801–817, 1939) made some highly interesting observations concerning the latency of this infection. I have recently extended some aspects of his work with the following results.

I find that LCM virus injected intracerebrally into adult mice always kills them with convulsions; injected intraperitoneally, it gives a disease from which they recover and are immune. However, after intracerebral injection of 1-day-old mice with LCM virus, one can induce regularly a symptom-free infection with as much virus present in the brain as there is normally at the height of a lethal infection. This condition persists for many months in a perfectly unchanged symptom-free situation. It seems to me, in this case, a tissue-culture approach could yield valuable information concerning the nature of the virus-host system, especially if a plaque technique could be devised for precise assay of the virus. Such an assay procedure applied to suspensions of the animal's cells, blood, etc., should reveal whether virus is continually liberated from the cells or perhaps occasionally liberated from some of the cells. This type of latent infection could then be investigated according to both methods outlined above. Such a model might lend itself to interesting investigation with the second method suggested, the study of intracellular physiology, particularly concerning self-duplicating particles. What is this virus doing under these circumstances? How is it held down by the cellular mechanisms in such a way that it only multiplies at a constant rate, and without apparently damaging any cells? The virus gains entry to the cell and then multiplies, perhaps as sub-units; in order to function so smoothly and harmlessly, keying into the cell's biochemistry, these multiplying virus units must be very similar to normal replicating units present in the cell. Since this type of LCM infection appears to be harmless to the animal, it seems likely that the sort of control mechanisms, which I believe Dr. Lwoff was thinking of, and which regulate the number of these particles and their rate of multiplication, must operate here, preventing the unlimited multiplication of the virus and at the same time controlling the rate of increase

of the cell's normal cytoplasmic particles. In other cases of virus infection, no restraint is evident, and this may allow such a replicating virus particle to multiply so fast as to cause a cytopathogenic effect. An interesting speculation occurs to me: Could such an inhibitory control mechanism of particle replication rate be invoked to account for viral interference? Perhaps a small molecular by-product of the replication of the first virus can insert an inhibitory mechanism upon the superinfecting virus. The search for any such small molecular chemical involved in an interference phenomenon would have tremendous potential value as an inhibitor of virus multiplication. Leading to such viral antibiotics it would, of course, be well on the way toward finding a viral chemotherapeutic agent. Perhaps it is significant that the only agents which seem able to prevent virus infection are other viruses. In regard to the lymphocytic choriomeningitis virus, I feel it is perhaps possible to expand Dr. Lwoff's definition of a virus into the following: "A virus is something which sometimes does nothing."

Dr. Melnick: By confining my remarks to work in progress,[1] I hope they may serve to illustrate certain trends of research that have resulted from the impact of tissue culture on virology.

ECMO viruses.—It appears that more and more viruses will become recognized as we come to select the properly susceptible cell for the isolation of the new virus. Dr. Hull and his colleagues in Indianapolis have isolated a number of new viruses from monkey kidney tissues (*1*). This work has been confirmed and extended by Dr. Cheever in Pittsburgh and also in our laboratory by Mr. Riordan, who has examined the stools of 21 monkeys and isolated 25 viruses in monkey kidney cultures. Actually some of our monkeys were excreting 2 viruses at the same time, which accounts for the recovery rate of over 100 percent. The picture is further complicated by the fact that monkey kidney cultures often have latent virus infections, and the most common is foamy virus. As each virus group may manifest a typical morphological response, the distinctive cytopathologic response of 3 monkey viruses could sometimes be observed in a single culture: 2 of the viruses being monkey enteric agents added to the culture and the third being indigenous to the cells of the culture.

The enteric cytopathogenic monkey orphan, or ECMO, viruses that have been encountered in our laboratory may be divided into 3 groups based on the cellular changes produced during their infection of cultures. One group (A) produces the grapelike clusters of cells characteristic of adenoviruses. Further study by the complement-fixation reaction has shown their relationship to the human adenoviruses in that both groups of viruses share a common antigen. Another group of the ECMO viruses (B) produces rapid degeneration of at least two thirds of the cells in the culture—the culture then seems to recover and in a few days looks normal again. However, the typical epithelial cells are now replaced by a new type of long spindle-shaped cell. The new cells are now immune to challenge by the virus that caused the original cytolysis of the culture. However, the cells are susceptible to the ECMO viruses of group A, mentioned above, and of group C, which produces complete cell destruction with no recovery of the culture. A cooperative program between Dr. Hull's laboratory, Dr. Cheever's laboratory, and our own has been initated to determine the number of different viruses so far recovered and identified.

Applications of the plaque technique.—With Dr. G. D. Hsiung (*2*), we have adapted Dulbecco's technique to rubber-stoppered bottles. This has simplified the technique by eliminating the need of the humidified CO_2-air incubator, and has produced cultures that have a longer life under agar. Simple examination of the plaques can show us that we may be dealing with different groups of the enteric viruses.

Recently Dulbecco and Vogt (*3*) have found that plaque development among the poliovirus may be dependent upon the bicarbonate concentration, and Dr. Hsiung has been following this lead with the bottle technique. This test depends on a difference between virulent and attentuated strains in their rate of growth and size of plaques in high and low concentrations of bicarbonate ion: The attenuated strains seem to grow more slowly and produce relatively few plaques at low bicarbonate

[1] Aided by a grant from the National Foundation for Infantile Paralysis.

concentrations, whereas virulent strains produce approximately equal numbers of plaques at both low and high bicarbonate concentrations.

The virulent Mahoney strain of poliovirus seeded at the same dose into 4 bottle cultures, in which the agar overlay contained 0.1 to 0.9 gm. percent $NaHCO_3$, yielded the same plaque count in each series of bottles, although the plaques grew more slowly in the cultures with the lowest amount of bicarbonate. The attenuated Y-SK strain of poliovirus (*4*), seeded as above, failed to produce plaques at the lowest concentration of bicarbonate and, even at the next higher concentration, their appearance was delayed.

This technique should prove useful in the future for characterizing naturally occurring strains as well as for genetic studies. For example, a virus recovered from a fecal specimen (TC 512) was titrated at a high (0.45 gm. %) and low (0.075 gm. %) bicarbonate concentration. The virus at the high bicarbonate concentration produced over 70 plaques at TC fluid concentrations of 10^{-4}, 11 plaques at 10^{-5}, and 1 plaque at 10^{-6}. At the low bicarbonate concentration no plaques were produced even with TC fluid concentration of 10^{-2}. Thus the efficiency of plating (ratio of plaque-forming particles containing low bicarbonate to those in bottles with high bicarbonate) was $<10^{-4}$. Tests for virulence in monkeys showed that 10^5 virus particles inoculated into the brain failed to produce disease in them.

Strains found to be virulent for the monkey have had a high efficiency of plating. However, we have obtained data on other strains that have a high efficiency of plating at low bicarbonate concentrations, but with low virulence for monkeys. It is not possible to assess the significance of these observations until more is known of the correlation of the *in vitro* and *in vivo* results.

Dr. Hsiung has extended this work to the ECHO viruses also. The Farouk strain of ECHO-1 virus differs from poliovirus in producing plaques of equal size and in equal number at low and high bicarbonate concentrations.

ECHO viruses.—Tissue-culture methods have led virologists to the discovery of new groups of human viruses. For example, at least 19 new enteric viruses have been recognized within the past 5 years, and there are undoubtedly other types isolated (*5*). The ECHO viruses appear to fall into 2 groups on the basis of plaque morphology and cell susceptibility (*2*). Members of group A (types 1, 3, 4, 6′, 9, 11, 13 through 19) produce small, irregular-shaped plaques on rhesus but are negative on patas cultures. Group B ECHO viruses (types 7, 8, and 12) produce plaques, usually larger and more circular than those of group A, in both patas and rhesus cultures. While the prototype strains of types 2, 3, 5, and 6 have not produced plaques, they have been found to be cytopathogenic for rhesus but not for patas cells in tube cultures, and are considered to be members of group A. The ECHO viruses have been associated with aseptic meningitis cases, with or without rash, and those actually isolated from the spinal fluid of such patients have been members of group A.

The plaque technique is already proving advantageous in the diagnostic laboratory for identifying 2 viruses in a single specimen. Polioviruses grow much faster than most of the ECHO viruses and usually outgrow them when present in the same sample. However, if the specimen is tested by means of the plaque technique, particularly in bottles in which the sheets are maintained for a period of up to 2 weeks, the characteristic plaques of each virus can be distinguished and the progeny readily identified by serological tests.

Effect of cell nutrient on cytopathogenic response.—I would like to discuss a point made by Dr. Enders in connection with his work on the measles virus. All of us should pay more attention to the nutrition of the cell at the time it is infected. Recent work (*6*) has shown that, with measles virus in a human epidermoid cell line (Hep-2), 2 completely different morphological patterns of degeneration could be obtained depending on the composition of the nutrient medium employed for virus propagation.

Hep-2 cells were grown in Eagle's basal synthetic medium supplemented with 10 percent calf serum. Well-developed cell sheets (fig. 1, plate 68), were inoculated with the Edmonston strain of measles virus. At the time of inoculation, the growth medium was replaced with either fresh Eagle's medium containing 5 or 10 percent

calf serum or with the biological medium recommended by Enders, composed of 35 percent bovine amniotic fluid, 5 percent beef-embryo extract, 25 percent heated horse serum, and 35 percent Hanks' balanced salt solution. Cytopathic changes appeared from 2 to 14 days after virus infection, depending on the dose of virus inoculated. When Enders' medium was used as a nutrient, flat syncytial masses appeared in the inoculated cultures, as described in Dr. Enders' paper. Some of the giant cells contained several hundred nuclei (fig. 2, plate 68), and, in the last stages, the whole cell sheet of over 1 cm.^{2}, was transformed into a few huge syncytia. In the cultures nourished in Eagle's medium, the cells, after infection, became either elongated and spindle-shaped or rounded (fig. 3, plate 68). Very few polynucleated cells were present and most of them contained less than 10 nuclei each.

Evidence was obtained to show that a deficiency in the Enders' medium was a responsible factor in the appearance of multinucleated cells. When Enders' medium was supplemented with the amino acids, vitamins, and glutamine contained in Eagle's medium, or with glutamine alone, giant-cell formation was greatly reduced and most of the cells involved were typical of measles degeneration as in Eagle's medium alone. That the effect could be attributed primarily to glutamine was proved by its omission from the Eagle's medium. With this deficient medium, the appearance of infected cultures was very similar to that in Enders' medium (fig. 4, plate 68).

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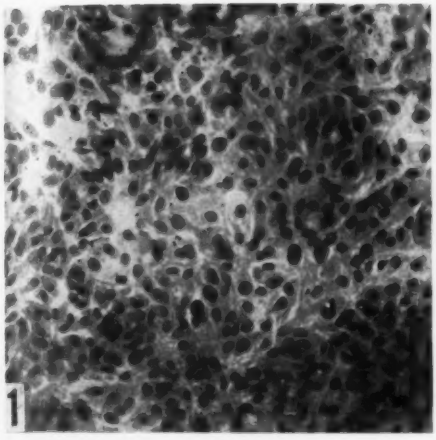
Plate 68

Figure 1.—Uninoculated culture of Hep-2 cells grown in Eagle's medium. Hematoxylin and eosin stain used throughout. ×150

Figure 2.—Measles-infected culture showing multinucleated giant cells. Culture maintained in Enders' medium after inoculation of virus. ×180

Figure 3.—Measles-infected culture maintained in complete Eagle's medium, 10 days after inoculation of virus. Cytopathic effects are widespread but are confined primarily to individual cells. ×180

Figure 4.—Measles-infected culture maintained in glutamine-deficient Eagle's medium from the time of virus inoculation. ×160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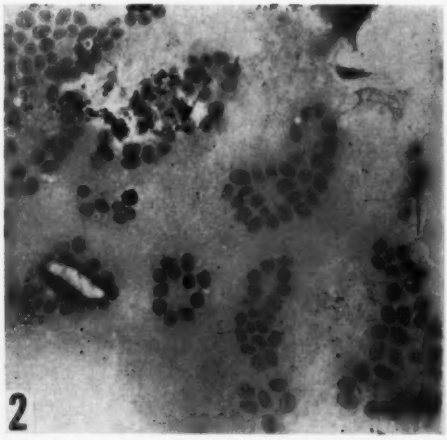
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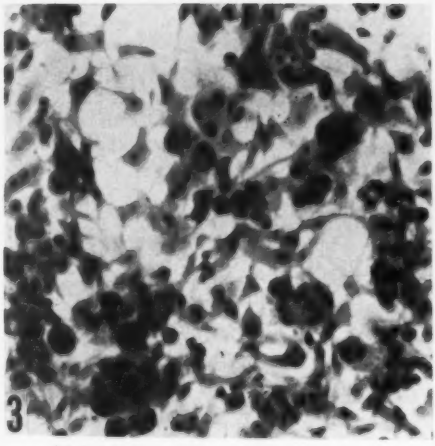
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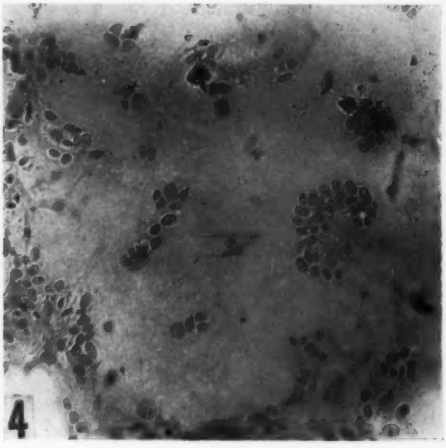
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Dr. Hull: I would like to comment on Dr. Melnick's presentation. In his first slide, Dr. Melnick has shown a very good example of simian virus C.P.E., group I. It is possible that a little discussion between us might determine which agent it is. Figure 3 had a cytopathogenetic effect characteristic of $S.V._6$ and the description of its healing properties are in keeping with our observations on $S.V._6$.

The observation on the effect of *p*H or alkaline conditions was extremely interesting to me and I am sure it is to other members of the industry who are currently working with the poliomyelitis vaccine. One of our colleagues, some time ago, observed that when the *p*H of the medium was raised a virus that previously was either missed or developed very late in the incubation of cultures could be isolated. I am sure that most people who have worked with poliovirus realize that when it is partially or improperly inactivated by formalin, it behaves differently from virus that is merely diluted to the same level of infectivity. However, recent work, by several of us, on this problem has shown that if the combination of medium and vaccine was raised to *p*H 9.2 before it was introduced into the culture, these viruses, partially inactivated by formalin, which previously would not show up until 20 or 28 days, could now be demonstrated much sooner, some within 2 or 3 days. From our experimental studies, we have been able to find viruses that we had missed entirely at *p*H 7.4. We did not necessarily associate this with avirulence. I hoped that you might have something further to say on this point. Certainly we have evidence that one partially inactivated virus was not avirulent but perhaps the high *p*H might have aided in its detection. I wonder if you would care to say anything further about the relationship between attenuated virus and partially inactivated virus in respect to how they react at different *p*H levels?

Dr. Melnick: I would like to emphasize again that it was Dulbecco and Vogt who discovered the correlation between plating efficiency at varying bicarbonate concentrations and virulence of poliovirus. Virulent viruses grow readily at high as well as low bicarbonate concentrations whereas attenuated strains grow poorly at low bicarbonate concentrations.

The phenomenon mentioned by Dr. Hull—that poliovirus particles which are partially inactivated, or associated with inactivated particles, may be detected earlier in the presence of an alkaline medium—might be the influence of alkali on dissociating aggregates of viruses. An aggregate with a core of active virus might be dissociated at an alkaline *p*H and liberate active virus from the center of the aggregate. This might be the reason why Dr. Syverton, and others, were able to isolate virus from certain vaccine preparations by means of tests in monkeys when others were unable to do so by tests in tissue culture. Here, again, the animal, by means of phagocytosis or some other process, might have digested away some inactive virus from the outside of an aggregate containing active virus in its center. The tissue-culture cells would come in contact only with inactive particles on the surface of the aggregate, which would be an effective barrier to the initiation of infection.

Dr. Kehr: As indicated by Dr. Black, I should like to illustrate a practical use of tissue culture in plant pathology. The technique deals with the eradication of potato virus X from varieties that are universally infected with the virus.

Potato virus X was first described in 1925. At that time, many of the older potato varieties were already universally infected with the virus, so that today no clones of many of these varieties are available free of virus. This is of great economic importance because it has been demonstrated that virus X tends to reduce the yields of potatoes from 5 to 20 percent, depending upon the person who performed the experiment.

It has been proved by many persons that a meristem tissue tends to be lower in titer of a virus than the tissue directly below the meristem. A technique based upon this fact was first suggested by Norris in Australia, and it will be described here.

Tissue from the upper 1 mm. of the bud meristem was removed from a rapidly growing plant, sterilized, and placed on White's medium containing supplements of indoleacetic acid, copper, and molybdenum. Such excised meristems eventually formed callus, which in turn differentiated into stem and root tissues. Subcuttings

may be made at any stem internode and recultured as many times as desired. In this way the titer of the virus tends to become lower and lower.

At this point, another technique may be introduced. Substances such as malachite green, thiouracil, or 2,6-diaminopurine have been shown to inhibit the growth of viruses. These are introduced in the substrate as an additional supplement. In this way the virus has 2 strikes against it, so to speak, in that not only will the titer become lower as you subculture the potato tissue but the malachite green at a concentration of about 2 p.p.m. in the substrate tends further to decrease the growth of the virus. By this process it has become possible to isolate clones that are entirely free of virus X.

Dr. Potter: Dr. Syverton tells me that the quantity of virus is no longer an issue nor the quantity of cells, and that enough can be obtained for experiments on the acid-soluble constituents. I would like to hazard a guess as to what is happening in some of these cultures. We have demonstrated that, when a nucleotide precursor goes into what we call the acid-soluble pool, it goes into the entire pool of coenzymes that contain the same variety of pyrimidines, and then these coenzymes do not have any other exits except the nucleotides that are precursors for the nucleic acids. I am particularly interested in these cytopathology studies that show dissolution of the cell walls to form syncytia when certain viruses are present. It seems to me very possible in this situation that one has a balance of pulls in the direction of the various nucleoproteins, which results in the depletion of the particular coenzymes which are necessary for the maintenance of the cell wall. At this particular time, the best guess one can make is that this includes the uridine coenzymes. You will recall that in the presence of penicillin, there is a block in the metabolism of the uridine coenzymes with some interference in the formation of cell walls.

Maintenance of the material in the cell wall may depend upon the maintenance of the coenzymes that are necessary for the element in the cell wall. If one could take a large enough culture, infect it with a virus, and compare it with a noninfected culture, one would find that each particular virus tends to deplete the coenzyme pool of the cells in its own particular way. I just wonder whether anyone is attempting to separate these nucleotides? This would be quite feasible because the compounds you are dealing with would be compounds that, by and large, are not present in the medium.

Dr. Morel: I would just like to point out that the technique of growing the apical meristems of plants to obtain clones free from virus has been very extensively used in our laboratory since 1950. We have been able not only to obtain plants of potato free from virus Y and virus X but also some other ornamental plants such as dahlia, free from dahlia mosaic and spotted wilt, and carnations, free from several mosaics of carnation, etc.

Dr. Melnick: Monkey kidney cells often contain a latent virus that produces foamy degeneration, an observation first made by Dr. Rustigian and by Dr. Enders. The foamy virus can be passed in HeLa cultures and can be titrated in this way. This foamy type of lesion, however, is markedly different from the measles virus lesion, in that no inclusion bodies are present in the giant cells produced by foamy virus. Thus the presence or absence of intranuclear-inclusion bodies is a helpful determinant in deciding whether one is dealing with a measles-like virus or a foamy virus.

Dr. White: I would like to add to Dr. Morel's suggestion. In 1934, we were able to recover clones free of virus from infected clones of tomato roots in which the virus was highly virulent and therefore localized. Viruses of low virulence tend to become systemic and we could not recover virus-free clones from roots infected with those viruses.

Dr. Leighton: I am sure that all of us who work with tissue fragments in organ cultures or in sponge-matrix tissue culture have wondered why the virologists have not extended their studies to organized aggregates of cells. I would like to illustrate some of the kinds of organ patterns that one can see in sponge matrix. Chick embryonic liver produces a highly organized tissue in 2 to 4 weeks, which includes tubular-epithelial structures as well as spaces lined by endothelium. Perhaps in the case of hepatitis virus, work with embryonic liver of the appropriate species might provide interesting results. Cultures of human fetal brain give rise to a dense outgrowth of

glial tissue in 3 or 4 weeks. Adult human endometrium cultured in sponge matrix forms an "endometrial organism" (fig. 6, plate 65). The section is from a 17-day-old culture. Some glands have survived as well as some of the stromal cells. I would welcome the comments of a virologist on the possibility of working with more complex tissues *in vitro* to study some of their problems.

Dr. Bang: This is what I was trying to do last year, while working at the National Institute for Medical Research in England, with help from Dr. Fell at the Strangeways. It is true that there are all sorts of potential uses for this type of culture in studies of viruses. Human tracheal, bronchial, and nasal mucosa can be grown in just such an organized way, so that for the 2 months of our crude kind of culturing they remain ciliated and mucous secreting. Skin will remain skin and can be infected with the virus of herpes. A little vesicle appears just as it does in the intact disease (Bang, F. B., and Niven, J.). The nasal mucosa of a ferret may be infected in the same way, so that the top layer of the mucosa is stripped off by the action of the virus. The antibodies apparently disappear rapidly enough so that this method can be used shortly after the mucosa has been explanted. I think a number of us have been aware of this theoretical possibility in hepatitis. We don't know just what kind of a model to use.

Dr. Boll: I was interested in the observations on virus-free potatoes and virus-free plants. I wonder whether you or evolution removed the virus! In some work with Dr. Joanne Parker at the University of Texas, we found that we could, with the appropriate treatment, obtain shoots from fragments of an excised tomato root. We grew these small plants into plants in the field, and they looked very much like the strain from which the clone was originally derived. Unfortunately, the plants that we got from the root could not be crossed in any way. They were not interfertile, and they couldn't be crossed with the strain from which they were derived. In other words, we obtained a new plant. I wonder, in fact, whether your plants are the same plants as those with which you started?

Dr. Morel: We have raised several dozen potatoes and about 25 dahlias in this way and they look exactly the same.

Dr. Earle: The question arose whether immunologic characteristics were associated with the stroma or with the cells. On this point we have certain data, which I think you might find of interest.

Strain L mouse fibroblasts originated from a strain C3H mouse, an Andervont substrain of a 100-day-old adult. These cells have now been grown in a species-alien culture fluid for more than 16 years. This cell is free from all stroma, yet it is, immunologically, still definitely and without question a mouse cell. When these strain L cells from this C3H strain mouse are reinjected into mice of other strains, they do not grow. Over the period of years the ability of this strain to give rise to sarcoma when reinjected even into its own strain of mice gradually dropped from more than 66 percent to less than 1 percent. A low percentage of sarcoma is shown, even now, by the cell strain after it has been cloned for more than 5 years, and after the strain has been growing in alien species media for more than 16 years. When cultures of the clone are injected into heavily irradiated mice, the percentage of injections that originate sarcomas rises to 64 percent. If these irradiated mice have been previously immunized by injection with clone L cells, no tumors develop. It appears that the previous injections increase the resistance to the reinjections. When a sarcoma has been obtained, it can be subinoculated from mouse to mouse but with erratic subinoculation results. One experiment gave a line that after several passages reached 100 percent of incidence of sarcoma production. From these and other immunologic studies not detailed here, there seems no question that after more than 16 years in an entirely alien medium, these are still mouse cells.

Tissue Culture as a Tool for Studying the Development of Autonomy in Neoplastic Plant Cells [1, 2]

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Perhaps the most distinctive characteristic of a tumor cell is its capacity for uncontrolled or autonomous growth within the organism, animal or plant, in which it is developing. Without this there obviously would be no tumors. The question that we should like to concern ourselves with at this time, therefore, is: Why does one type of tumor cell, the crown-gall tumor cell of plants, proliferate independently of the morphogenetic restraints that govern so precisely the growth of normal cells? The uncontrolled growth of tumor cells generally appears to reflect a deranged metabolism in those cells. An understanding of the problem of autonomy will, in all likelihood, therefore, depend upon a characterization of the biochemical lesion(s) that is responsible for this altered metabolic state. Since tumor cells appear somehow to have acquired the capacity for autonomous growth as a result of uncontrolled growth and division, an attempt has been made to explore this problem through an analysis of the factors concerned with growth and cell division.

Growth of an organism generally results either from an enlargement of its constituent cells or from the combined processes of cell enlargement and cell division. These 2 processes appear to be dependent for their development upon specific substances that are synthesized by plant cells themselves. The growth-substance synthesizing systems concerned with the production of cell-enlargement and cell-division factors are, however, precisely regulated in normal plant cells. Since both of these substances appear to be essential for growth and cell division, an attempt was made to investigate the fate of these 2 as well as several other growth-substance synthesizing systems during and following the conversion of normal plant cells to tumor cells.

It has been found (*1*) that cells of normal tobacco-pith parenchymal tissue enlarge greatly but do not divide in the presence of growth substances of the auxin type. When, in addition to an auxin, a cell-division factor is applied, profuse growth and cell division result (*1*). Cells treated only with a cell-division factor neither enlarge nor divide. The

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[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8–12, 1956.

tobacco-pith cells have apparently lost, as a result of their maturation, a capacity to produce physiologically effective amounts of the 2 growth factors. A method is thus available for studying cell enlargement and cell division as separate processes in the same tissue.

Crown-gall tumor tissue has been shown to be a rich source of both a cell-enlargement (*3–6*) and a cell-division factor (*2*, *7*). It was found (*2*), moreover, that when these 2 growth substances were incorporated in a culture medium on which fragments of tobacco pith were planted, such tissue grew actively and showed, after a period of 1 month, a striking morphological and histological resemblance to crown-gall tumor tissue of tobacco. The artificially stimulated pith cells are, however, self-limiting and when they are planted on unsupplemented White's basic culture medium, their growth promptly stops. Crown-gall tumor tissue is, on the other hand, autonomous and is itself capable of synthesizing all growth factors needed for its continued abnormal growth on this relatively simple, chemically defined medium.

Recent studies (*8*) have shown, furthermore, that, as a result of the alteration of normal tobacco-pith cells to crown-gall tumor cells, the affected cells acquire the capacity to synthesize greater than regulatory amounts of both the cell-enlargement and cell-division factors. Whereas normal mature pith cells were unable to produce physiologically effective concentrations of either growth factor prior to their alteration to tumor cells, both substances were produced following transformation. If both growth-substance synthesizing systems had not been activated, continued growth and cell division and, hence, tumor formation would not have occurred in the test system used in these experiments. Thus, we see that an essential difference between the normal tobacco-pith cell and the crown-gall tumor cell appears to be concerned, at a physiological level, with the permanent unblocking of 2 essential growth-substance synthesizing systems concerned specifically with growth accompanied by cell division.

In attempting to characterize further essential growth factors concerned with the rapid autonomous growth of crown-gall tumor cells, an entirely different test system was employed. It has been found (*9–11*) that the alteration of normal plant cells to crown-gall tumor cells takes place gradually. This process can, moreover, be very accurately controlled experimentally. When, for example, the tumor-inducing principle responsible for the alteration of normal cells to crown-gall tumor cells acts on susceptible host cells throughout a 4-day period, rapidly growing, potentially malignant tumors result. When, on the other hand, this principle is allowed to act for only 34 hours before being inactivated selectively by a thermal treatment, small, slow-growing benign tumors are initiated. A 50-hour exposure of susceptible cells to the tumor-inducing principle results in tumors that grow in the host and in culture at a moderately fast rate. A similar type of tumor can also be obtained by allowing the tumor-inducing principle elaborated by a moderately virulent strain of the inciting bacterium to act on host cells throughout a 4-day period.

Sterile tissues isolated from 3 types of tumors similar to those described above and planted on White's basic medium have maintained their characteristic growth patterns in culture for periods up to 10 years. The relative growth rates of these tumor tissues on White's basic culture medium are shown in figure 1—A, B, C; plate 69. Since this material, which showed varying degrees of neoplastic change, was derived from the same plant species, it was admirably suited for a further characterization of the factor, or factors, that are limiting for rapid autonomous growth. In these studies the rapid-growing, fully altered tumor cell was used as the standard. This cell type can apparently synthesize in optimal or near-optimal amounts all of the growth factors needed for its continued rapid proliferation. The moderately fast-growing tumor cell required, in addition to the cell-enlargement factor (naphthaleneacetic acid), the vitamin meso-inositol, as well as glutamine and asparagine, to achieve a growth rate comparable to that of the fully altered tumor cell. Glutamine and asparagine were replaceable in this system by glutamic and aspartic acids.

It is interesting to note that a cell-division factor is not one of the growth factors that is limiting for the rapid growth of this type of tumor cell. The enriched but chemically defined basic culture medium does not contain this factor, yet these tumor cells proliferate very actively on this medium. Since an area of irritation resulting from a wound is essential if tumors are to be initiated in crown gall (*12*), it might be postulated that the cell-division factor synthesizing system is activated during normal wound healing. Following the transformation of normal cells to tumor cells, which occurs during this healing period, this system continues to be functional, indefinitely, as a result of the production by the altered cell of greater than regulatory amounts of the cell enlargement as well as the other growth factors described above. Alternatively, one might postulate that the cell-division factor synthesizing system is very lightly blocked in normal cells and that a low degree of cellular change resulting in a partial activation of the other growth-substance synthesizing systems is sufficient to permit the full activation of this system. The nutritional requirements necessary to increase the growth level of cells altered in a 34-hour period to that of fully altered cells appear to be somewhat more exacting than are those required to accomplish this end when the moderately fast-growing clone of tumor cells is used as the test object. Preliminary studies indicate that in addition to the cell-enlargement factor, meso-inositol, glutamine, and asparagine, at least 1 and perhaps several other factors appear to be required to accomplish this end. The response of tissue of this normally slow-growing clone of tumor tissue to several growth factors is shown in figure 2, plate 69.

Although these studies are not yet complete, they do suggest that, while normal cells altered to tumor cells during a 4-day period are themselves capable of synthesizing optimal or near-optimal amounts of all growth factors needed for their continued rapid proliferation, cells trans-

formed in shorter periods have had their requirements for rapid growth only partially satisfied for several essential growth factors. It therefore follows that, as the crown-gall tumor cell becomes more autonomous, its growth requirements in terms of externally supplied growth factors become less exacting. It thus appears that the transition from the slow-growing to the rapid-growing tumor cell is a gradual one involving quantitative rather than qualitative changes.

The results described thus far indicate that several quite distinct metabolic systems are permanently activated as a result of the alteration of normal plant cells to tumor cells. This leads to the production by the affected cell of greater than regulatory amounts of these substances. The continued production of these substances by the altered cell in greater than regulatory amounts could, and probably does, account for the continued abnormal growth of the crown-gall tumor cell. Since several quite distinct growth-substance synthesizing systems appear to be activated gradually during the transition from the normal to the fully altered tumor cell, it appears that there is some as yet uncharacterized master reaction which is specifically but gradually unblocked by the tumor-inducing principle and which, once activated, not only accomplishes the unblocking of several growth-substance synthesizing systems but also determines the rate at which the entire series of metabolic events concerned with growth and cell division proceeds.

Normal cells of the type from which the 3 clones of tumor cells described above were derived do not grow on the basic medium, nor do they grow on that medium when it is supplemented with substances that have been shown to be essential for the rapid growth of tumor cells possessing a low grade of neoplastic change. While the difference between the slow-growing and rapid-growing tumor cell is of a quantitative nature since both can grow continuously, although at different rates, on the basic medium, the difference between the normal cell and the tumor cell is qualitative. While such a growth factor as 6-furfurylaminopurine, which is limiting for cell division in certain test systems, is not essential as a supplement for the continued rapid growth of any of the 3 clones of tumor tissue studied, such a factor is absolutely essential for the continued growth in culture of the normal cells. The requirement of 6-furfurylaminopurine or the naturally occurring equivalent of that substance for the continued growth of the normal cells in culture represents, then, a basic difference between a normal cell and a tumor cell. The normal cells, unlike the tumor cells, also possess an absolute requirement for a cell-enlargement factor (auxin) for their continued proliferation in culture. There is, in addition, a third not as yet completely characterized requirement for the rapid growth of normal cells which appears to be largely satisfied by a combination of guanylic and cytidylic acids. Although the minimal requirements for the continued rapid growth of normal cells have not as yet been defined, these studies have progressed sufficiently to indicate that it is possible for a cell to acquire the capacity for autonomous growth as a result of the permanent unblocking of sev-

eral growth-substance synthesizing systems, the products of which are concerned specifically with growth accompanied by cell division. When the minimal requirements for the rapid growth of normal cells have been completely defined, it will in all likelihood provide the clue necessary to establish the metabolic system(s) specifically affected by the tumor-inducing principle and thereby provide the key to an understanding of the basic difference between a normal cell and a tumor cell.

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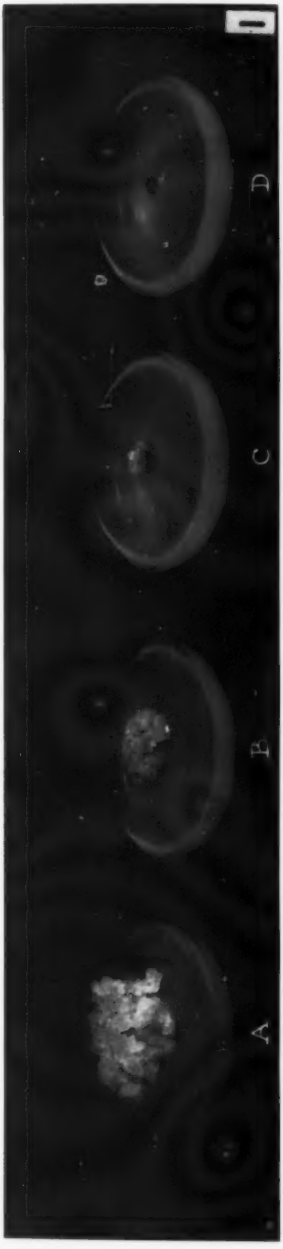
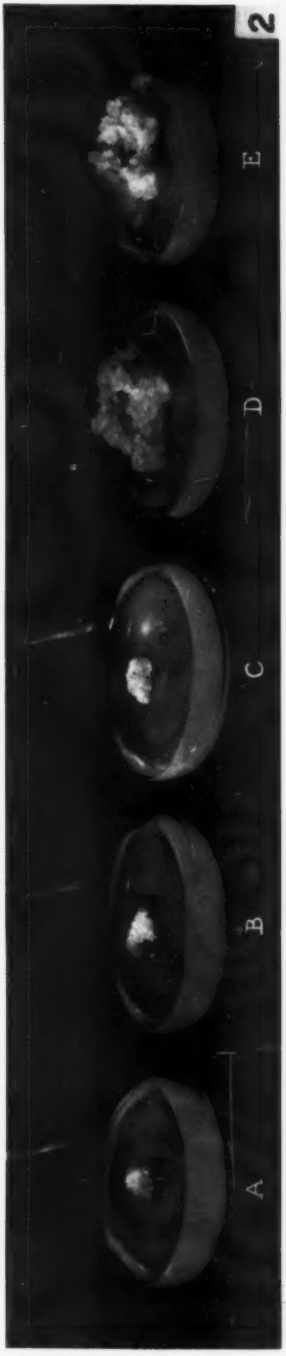
PLATE 69

FIGURE 1.—Relative growth rates of 3 clones of crown-gall tumor tissue that show different degrees of neoplastic change, planted on White's basic culture medium: *A*, fully altered, rapid-growing tumor cells; *B*, partially altered moderately fast-growing tumor cells; *C*, slightly altered slow-growing tumor cells; *D*, normal cells of the type from which the tumor cells were derived. Normal cells of this type do not grow on the basic culture medium.

FIGURE 2.—Slow-growing tumor cells possessing a very low grade of neoplastic change planted on: *A*, White's basic culture medium; *B*, basic medium supplemented with naphthaleneacetic acid, a cell-enlargement factor; *C*, basic medium supplemented with 6-furfurylaminopurine, a cell-division factor; *D*, basic medium supplemented with yeast extract, casein hydrolysate, and naphthaleneacetic acid; *E*, fully altered tumor cells planted on the unsupplemented basic medium.

Photographs by J. A. Carlile.





Braun

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DISCUSSION

Dr. Morel: Dr. Braun stated that, as far as plant tumors are concerned, the one biological characteristic that distinguishes tumor cells from normal cells is the capacity for uncontrolled growth of the tumor cell within the organism. We all agree with that statement, and in most cases this autonomy is easily demonstrated by grafting experiments. Dr. White showed, a long time ago, that tumor tissues grafted into a suitable host, even after a long period of cultivation *in vitro*, will continue to grow in an uncontrolled manner within that host. Disorganized growth *in vitro* cannot in itself be taken as a criterion for tumors. Some plant tissue, such as carrot parenchyma, will grow *in vitro* apparently in a disorganized manner without any growth factor added to the medium; others, such, as artichoke tuber, with only auxin added to the medium; still others, such as the tomato stem will grow in that manner only with the 2 growth factors indicated by Dr. Braun—cell-division factors, such as kinetin or those factors present in coconut milk and auxin. Even in the same plant, it is possible to find different tissues that will respond to growth factors as the different kinds of tumor mentioned by Dr. Braun. For example, embryonic tissue from the hypocotyl of *Lupinus* will grow *in vitro* at the same rate as tumor tissue without any growth factor added to the medium, but tissue taken from the adult plant will grow only if both growth factors are added. The same is true of embryonic tissue of *Pinus strobus*. So far as growth is concerned, embryonic tissues are comparable to tumors, but by aging they lose this character. I think other qualities than the differences in growth rate or the growth factor are still more characteristic of tumor tissue. May I remind you that one of the most important differences from the biochemical point of view, between normal and tumor tissue was that found by Camus, Wildman, and Bonner. By electrophoresis, they were able to show that in the cytoplasm of the tumor cell there is always a very large molecule of protein never found in normal cells. Recently, in Paris, Lioret, making a survey of free amino acids in various crown-gall tissues cultivated *in vitro*, found consistently in the crown-gall tissue an unknown amino acid that wasn't present in normal tissue. I have studied the metabolism of arginine in normal and tumor tissue of the artichoke. During the rest period in winter, arginine constitutes about 80 percent of the soluble nitrogenous material. If a piece of tissue is taken during the resting period and is put into culture, the arginine disappears very quickly. After 6 days of culture, the arginine is almost entirely transformed into proline, glutamine, and hydroxyproline. The arginine is a good source of nitrogen for this tissue, and it is possible to grow such tissue on a medium containing only arginine as a nitrogen source. When we do this with normal tissue, the arginine is quickly metabolized into other amino acids. If we do the same experiment with tumor tissue of the artichoke, we see first that the arginine never disappears entirely as it does in normal parenchyma. Besides arginine, we found 7 other compounds that, although mostly still unidentified, all give the Sakaguchi reaction. We were able to identify 2, γ-guanidobutyric acid and α-hydroxy-δ-guanidovaleric acid. I think such studies on the metabolism of tumor tissue might give a clue to the basic differences that exist between the normal and tumor tissue.

Dr. Black: It is always a pleasure to read Dr. Braun's reports of his beautiful experiments on crown gall and this particular paper is no exception. Dr. Braun has shown that crown-gall extract, plus a high concentration of naphthaleneacetic acid, gives an unorganized type of tumor, whereas crown-gall extract plus a low concentration of naphthaleneacetic acid results in teratomas. It is known from earlier experiments that living crown-gall tissue gives off auxins that have effects on healthy cells. The question therefore arises, does living crown-gall tissue give off these various growth

substances that have been demonstrated in the extract? Apparently these growth substances can induce healthy cells to act like crown-gall cells as long as they are supplied to the healthy cells. Therefore, it seems possible that individual crown-gall cells could have the effect of making neighboring healthy cells behave like crown-gall cells. This possibility has a bearing on the interpretation of facts convincingly demonstrated in previous experiments by Dr. Braun. The recovery of teratomas was interpreted on the basis that all the cells in the teratoma were identical, tumorwise, and that the individual cells were being cured. This interpretation was supported by an experiment in which Dr. Braun grew these tumors on Dr. White's medium plus indoleacetic acid. In this case one would expect that, if the teratomas were caused by a mixture of crown-gall and healthy cells, the indoleacetic acid would inhibit the crown-gall cells and stimulate the healthy cells, and so result in a selection of healthy cells. In other cases the teratomas were grown on White's medium without added indoleacetic acid, and under these circumstances one would expect a selection of crown-gall cells from a mixture. However, it seems to me that Dr. Braun's paper raises the possibility of a stable mixture of healthy and crown-gall cells being maintained by the individual crown-gall cells giving out these substances, which make neighboring cells behave like crown-gall cells. The same possibility, it seems to me, has a bearing on Dr. Braun's work showing that one can get a graded series of crown-gall tumors of different proliferative vigor, depending on the length of time that the bacteria have been acting on susceptible cells. This has been interpreted on the basis that each such tumor is made up of cells that all have the same proliferative ability, although the proliferative level varies from tumor to tumor. Here again we have the same possibility that if, as Braun has just shown, the crown-gall cell is producing various growth substances, it would be possible to have a mixture of crown-gall cells and healthy cells producing a given pattern because crown-gall cells might act on neighboring cells that are not really crown-gall cells. The problem has a bearing on the virus hypothesis of crown-gall incitation. Dr. Braun postulates that a master reaction may occur in crown-gall cells. Could this master reaction be controlled by a master reactor, which is a virus? Dr. Riker said just enough about the technique of isolating individual cells to tantalize me with the possibility that the technique could be applied to Dr. Braun's teratomas, and to the aforementioned graded series of tumors of different proliferative vigor, to determine whether they are mixtures of healthy cells and crown-gall cells. The results might be relevant to the virus hypothesis. If one were to isolate only crown-gall cells and healthy cells, the result would be compatible with the virus hypothesis. If, on the other hand, one were to obtain a series of tumor growths, derived from individual cells, which formed a gradient in respect to proliferative vigor, the result would appear to require the postulation of an improbable system of virus strains to accommodate the virus hypothesis.

Dr. Kehr: After the thought-provoking papers concerning the role of bacterial viruses as possible tumor-inducing principles of crown gall, it seems especially important to review critically the evidence for a genetic origin or control of genetic tumors.

Genetic tumors may be defined as tumors that arise spontaneously and are incited only by factors from within the individual itself. Such tumors are independent, and require no environmental agent for their inception. However, once the plant cells become tumorous in nature, they behave in much the same fashion as plant tumors of other types in that they have the capacity for uncontrolled or autonomous growth, and once the alteration from normal cell to tumor cell has been completed, the affected cells lose their ability to revert to permanently normal cells. In short, they differ from most other plant tumors primarily in the manner they are incited.

Genetic tumors may be observed when, for example, 2 species of *Nicotiana*, *N. langsdorffii* and *N. glauca*, both of which are in themselves always normal in their growth, are hybridized. The subsequent F_1 hybrids show tumorous growths in 100 percent of the population, with never an exception to date. Such tumors may arise on roots, stems, or leaves. Perhaps there are present those who would like to consider these changes as mutations.

Explanations of the causal agent involved in such tumors have ranged from cyto-

plasmic factors by Whitaker, to chimeral hybrid tissue by Kostoff, to hyperauxiny by Kehr and Smith. All of these theories may be rejected on one basis or another.

The evidence for a genetic origin rests upon the following observations. If we represent the 2 genomes of *N. glauca* by "GG," each "G" representing 1 complete set of 12 *glauca* chromosomes and "LL" as the chromosome complement of *N. langsdorffii*, each "L" representing a total of 9 chromosomes from *langsdorffii*, the F_1 hybrid would then have a genomic constitution of "GL." These, as I have already indicated, produced tumors in 100 percent of the population. Unfortunately, the F_1 hybrid is sterile and cannot be used in further genetic work. By the use of colchicine, however, a doubled hybrid or amphidiploid may be produced, which, to all intents and purposes, is identical to the hybrid in that the amphidiploid also produces tumors in 100 percent of the population. However, here we have a plant that now becomes fertile and produces seed; the seed upon planting produce plants that form tumors; the character is therefore inherited through the seed. If we start again with the amphidiploid, "GGLL," and cross it with the *langsdorffii* parent we get a triploid with the genomic constitution of "GLL." This again produces tumors in 100 percent of the cases. If this process is repeated once more, crossing the tripoloid with the *langsdorffii* parent, we obtain a population in which we get essentially *langsdorffii* plants with 1 or more chromosomes of *glauca*. When we get plants of this nature, we no longer find tumors in the population. In other words, these plants have now lost their ability to produce tumors. For tumors to be formed, we must have at least a full complement of chromosomes from both of the parental species. The original cross may be made either way, it may be made with *glauca* as male or female. Below is a diagrammatic sketch of the crosses described:

GG (*N. glauca*) × LL (*N. langsdorffii*)
↓
GL (tumors—but sterile)
↓
plus colchicine
↓
GGLL (tumors—produces seed) × LL
↓
GLL (tumors) × LL
↓
LL plus segregating chromosomes of *N. glauca* (no tumors)

During the course of this Conference the question arose as to whether greater emphasis should not be placed upon growth-regulating substances with large molecules in which there is either little or no free movement. We know in connection with these genetic tumors that grafting one species upon the other, reciprocally, as well as upon the hybrid, there is no movement of the factors producing abnormal growth through the graft union. For the tumor condition to arise, at least 1 set of chromosomes of each species must be incorporated within the same cell. The genomic balance is unimportant, thus these combinations have been obtained that produce tumors equally well:

GL
GGL
GGGL
GLL
} All produce tumors equally well.

Small fragments excised from young plants, which appear normal in growth, when placed in tissue culture form tumor tissue *in vitro*. In other words, the tumor-inducing factor is produced autonomously within small fragments of excised tissue and does not require a transport of elaborated substance to be localized in the tumorous area from elsewhere in the plant.

As indicated in Dr. Braun's paper, his crown-gall tissue derived from *Nicotiana tabacum* pith cells required 3 growth factors:

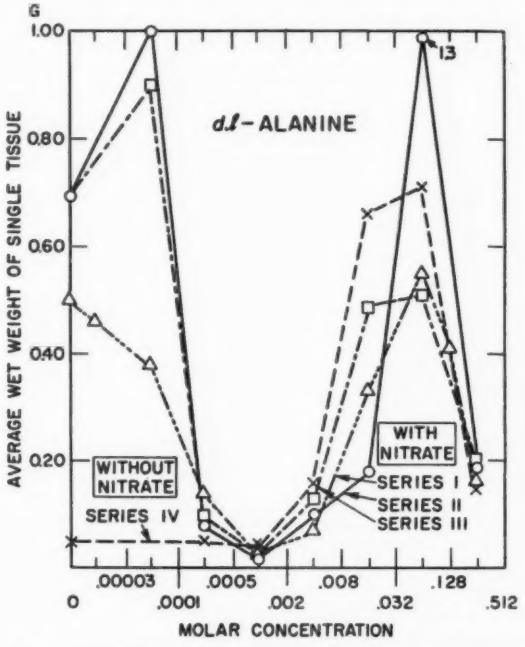
(1) A cell-enlargement factor—or auxin
(2) A cell-division factor—or kinetin
(3) An unknown factor from yeast extract and inositol

In the process of evolution, plant species, by the process of natural selection, have developed adequate enzyme systems for the production of factors for cell enlargement and division. It would be expected that both parental species involved in genetic tumors therefore would have adequate amounts of both auxin and kinetin. If, in the case of the third factor, it could be demonstrated that its inheritance was controlled quantitatively by complimentary alleles, part of which were carried by each of the parents involved, the nature of genetic tumors and the inducing principle would remain less obscure. Our own experiments have shown that *N. glauca* seedlings grown in sterile culture on substrate containing yeast extract or coconut milk develop abnormal growth, *but this growth is not typical of tumor tissue.* In other words there remains yet an additional unknown that in crown-gall work is termed "tumor-inducing principle."

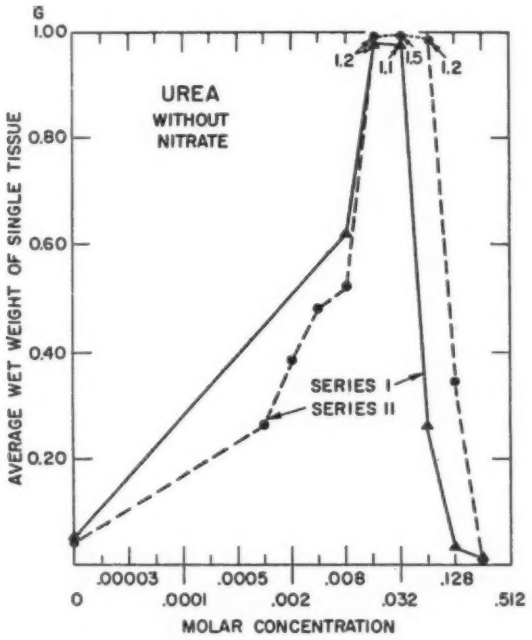
Dr. Riker: With this excellent paper, we have added reason for being proud that Dr. Braun received his Ph.D. degree with us at Wisconsin. Finding nothing particular to argue about in his presentation, perhaps I may comment on some related work.

With tissue cultures, Dr. Hildebrandt and I have accepted the opportunity to use bacteriological techniques to determine what metabolic substances encourage growth, what inhibit growth, and at what dilutions.

If a substance should be used to encourage growth, there must be satisfactory



TEXT-FIGURE 1.—Average wet weight of sunflower tissue cultures grown on synthetic media with different concentrations of urea as the sole nitrogen source.



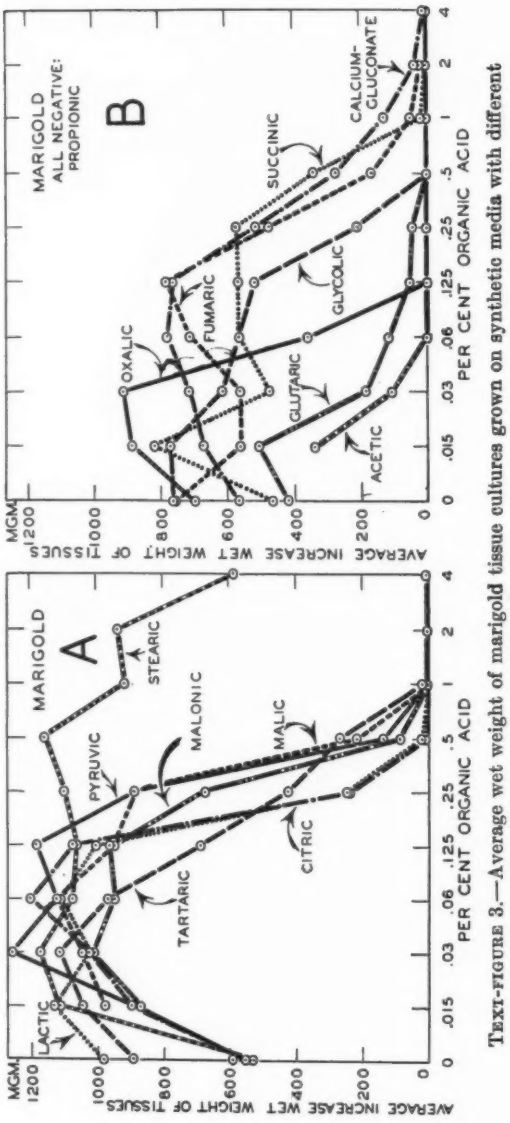
TEXT-FIGURE 2.—Average wet weight of sunflower tissue cultures grown on synthetic media with different concentrations of *dl*-alanine either with or without nitrate.

permeability and enzymatic action. Furthermore, the metabolic products must not be inhibiting. Much of our work has been concerned with the way various tissues in culture either used, tolerated, or were inhibited by common metabolic substances. From an extensive collection of data, 3 figures may illustrate certain critical items.

The importance of the amount of the metabolite sometimes has been overlooked in the emphasis on different substances and differences between tissues. When urea was used as a source of nitrogen, poor growth developed either with too little or too much. The right amount had a narrow range, as shown in the sharp peak in text-figure 1. As in all these examples, the weight is indicated on the left side and the geometric progression in amount across the bottom. All tests were made at approximately *p*H 6.0, which was favorable.

The situation with alanine was of special interest. As with all of many amino acids tried, except arginine, growth in an otherwise favorable medium was inhibited at about 0.001 *M* dilutions. No growth appeared with larger amounts of other amino acids except alanine (text-fig. 2), glutamic acid, and aspartic acid. With these 3 at 0.064 *M*, growth was fair to excellent. The frequent association of these 3 amino acids with transamination and the presence of transaminase helps to clarify these results.

Among the sources of carbon, we have examined a number of sugars and polysaccharides. Assuming that they were oxidized to alcohols and organic acids, a number of these common metabolites also were tried to see whether they encouraged growth or inhibited it. In text-figure 3, A and B, the results with marigold tissue and a number of organic acids at *p*H 6.0 have been recorded. What would have been good growth was inhibited by all dilutions of propionic acid used, and by high dilutions of acetic and glutaric acid, *viz*, 0.03 to 0.06 percent (text-fig. 3B). However, with high dilutions, *viz*, 0.015 to 0.125 percent, growth was increased slightly by a number of



TEXT-FIGURE 3.—Average wet weight of marigold tissue cultures grown on synthetic media with different concentrations of various common organic acids.

acids including citric, lactic, malic, malonic, tartaric, and pyruvic (text-fig. 3A). With all of these acids, growth was inhibited or stopped completely at 0.25 to 1.0 percent. Apparently, under these circumstances, relatively little propionic, glutaric, or acetic acid would have to be produced as metabolic products to prevent this tissue from further growth, and, perhaps, to kill certain cells. Somewhat larger amounts of various other acids also were inhibiting.

This question occurs: Can we develop a nutrient substance, which these diseased cells will take up and use differentially and from which the cells will produce an inhibiting metabolic product? In any event, many interesting experiments are suggested.

Perhaps I can mention that, as Dr. Black has reported, an impressive array of good men have tried to find a virus in crown gall and have failed. We also have failed, and, I believe, Dr. Braun has failed. For the time being, Dr. Black has convinced me pretty well that we have better use for our time than searching further for a virus in crown gall.

Dr. Steward: Since this question has already been raised I want to supplement what Dr. Morel has already said. It is true that hydroxyproline in the free state is rather rare in plants. But it has been isolated in quantity from certain species but only in the allo-form. It is also very dramatic that in every case we have studied, rapidly proliferating cells have in their protein an unusual amount of hydroxyproline. This has been isolated and characterized as to its stereoisomeric form. Thus, the presence of hydroxyproline in the protein is absolutely definite, and I think it is significant in relation to these observations on dividing cells that there is a large molecule, which may be indiffusible, associated with these activities.

May I add just 1 or 2 other points. I congratulate Dr. Morel and his associates on the identification of these guanido compounds. We also have observed certain basic substances in tissues and believe they are guanido compounds from certain reactions. What worries me in all this, however, is that we try to look for causal connections between this tumor-like activity and not results; the difficulty is to distinguish between some difference that is the result of this metabolism and something that is causally associated with it. We now know many differences between these rapidly dividing cells and their normal counterparts, not only metabolic differences but differences in constituents and in responses to enzyme inhibitors. It seems important to me to distinguish between the altered metabolism resulting from the growth-induction effects, and the basic conditions that cause growth in the first place. On this point one of the crucial needs is to find whether there is a "tumor-inducing principle" which has reality, is a simple molecule, and can be isolated. I have been keeping my ears cocked in the hope that this would come out in the discussion. We all now agree, perhaps, that cell division and cell enlargement can be controlled by opposing the effects of an auxin-like substance and a cell-division substance. But do we need something else? I know of many examples of differences between growing cells and nongrowing cells that could be added to those that have been mentioned. But I still look for something that may be, peculiarly, the tumor-inducing principle. I am reminded, in this connection, of a remark attributed to Bateson, who said, in the days when there was much controversy about genetics, that they had factors for every kind of cat—for black cats, white cats, and Manx cats, but they didn't have a factor for "catness." I would like to see whether there really is evidence for something in the underlying control of growth by cell division—the essential something on which the stimuli work and without which they alone could be of no avail.

Dr. Skoog: I would like to second Dr. Steward's statement. The only thing is to find out where and how these required factors are produced in autonomous cells and tissues. Dr. Braun's nice experiments show that there actually are discernible differences in synthetic capacities or intensities between normal and tumorous tissues.

I would like to ask Dr. Braun if all 18 amino acids were required, because we find that, for our strain of tobacco, tyrosine will do the same trick as the casein hydrolysate.

Dr. Braun: I tested them individually and found that no single one would replace the 18. I have not had the opportunity to test them in comparison with tyrosine.

Dr. Klein: Our laboratory has also been concerned with the nature of virulence in crown-gall bacteria. We have developed a quantitative bioassay for crown gall with discs of carrot. If we take a strain of bacteria, the B6 strain, and inoculate it into carrot under standardized conditions, we get approximately 100 mg. of tumor tissue in 15 days. We can use other strains of bacteria, such as S5-6, which will give about 50 mg. of tumor tissue under the same conditions, or H-100, which will give about 25 mg. of tumor tissue. Using the techniques of Dr. Braun, we started to play around with kinetin, naphthaleneacetic acid, and yeast extract—adding these components to the discs of carrot at various times during the development of the tumor. We found that the addition of a wide range of concentration of kinetin to discs inoculated with strain B6, the highly virulent strain, had no effect. However, when we added various concentrations of kinetin to discs inoculated with S5-6, we were able to increase the amount of tumor tissue formed from about 50 to 85 mg. Higher concentration of kinetin was required to raise the weight of the tumors induced with strain H-100 to the same level. These tests and others have indicated to us that each of the various steps in the process of tumor-cell formation involve the activation or, as Dr. Braun has said, the unmasking of enzyme systems, etc., that are responsible for the formation of one or more of the growth substances or for the maintenance of higher than regulatory amounts of growth substances found in a tumor cell. These preliminary results also indicate that one action, although not the only one, of the tumor-inducing principle, is the activation of a system synthesizing a compound whose biological effects may be mimicked by kinetin.

Dr. Gautheret: I think one of the many problems in regard to crown gall is to isolate the tumefacient factor. Dr. Braun's very interesting work can be considered as an attempt in this direction. His experiments show that there are several growth factors in the crown-gall tissue that do not exist in normal tissue but these factors do not promote the crown-gall transformation. The tissues treated by these growth factors are always normal. This sort of experiment is not very different from Dr. Skoog's experiments with kinetin or Dr. Steward's with coconut milk. Another sort of work consists of trying to extract the tumefacient factor from the bacteria, which is done by Dr. Klein and Dr. Manil. With extracts of bacteria, they obtained neoplasms that are similar to crown gall, but, as far as I know, the tumor properties of these neoplasms have not been tested. Perhaps it is only a new growth and not a tumor. However, the tumor transformation seems to be obtained without bacteria. de Ropp also obtained this transformation by grafting a piece of crown gall on a stem segment of sunflower. He believed that his newly formed tissue had the characteristics of a tumor. I know this idea was not accepted, and I tried, with Dr. Camus, to verify it on another kind of tissue. We confirmed the results. In our work we grafted a piece of crown-gall tissue on a root fragment of *Scorzonera*. A callus was induced; this callus was transferred *in vitro* on a medium without growth substance, and the growth was obtained exactly as in the crown-gall tissue. This sort of experiment, if confirmed, indicates that there may be a tumefacient agent in the tissue. But, if not confirmed, we cannot be sure that this exists and, of course, it is not necessary for a tumefacient factor to exist in the crown-gall tissue because, when the cells are altered by the bacteria, this alteration may be permanent and does not necessitate a permanent action by the tumefacient factor. Finally, it is possible to obtain such permanent transformations *in vitro*. In the phenomenon of "anergy," the cells are quite autonomous as are crown-gall cells. They can divide without growth substances and, upon grafting, they produce tumors. These tumors are sometimes slow-growing and at other times, for example, in tobacco, the growth obtained by grafting this anergized tissue is much more rapid than the growth obtained by true crown gall. In tobacco, it is almost a spontaneous transformation. The auxin does not act specifically, but only as an activating agent which favors the division of the cells. The transformation is another kind of action. This transformation is obtained so readily that it must not be compared to crown gall, although the kind of transformation may be the same.

Dr. Henderson: In the 2 cultures shown by Dr. Braun, when he only kept the crown-gall bacterium in the tissue a short time, the tissues did not grow so well as when the

bacteria had been there for a long time; the 36-hour explant *versus* the 4-day one. Is this a habituation process? Dr. Lwoff said, if just 1 or 2 of the molecules of permease could get into the cell, the rest of the process could go to completion. If habituation is an induction, can we grade it on a time basis? My conception is that if just 1 molecule got in, it was enough to give the 100 percent effect and not just a gradation. I am confused, Dr. Lwoff, by this slow-growing tissue and wonder whether we have here a type of habituation or of induction.

Dr. Lwoff: My impression is that Dr. Braun's results do not contradict the hypothesis of the induction of a steady state somehow analogous to the situation described by Melvin Cohn with *Escherichia coli* and β-galactosidase. We have to bear in mind that when a cell has been exposed for a few minutes to a given molecule, it may have acquired a new property that will be transmitted indefinitely under certain experimental conditions. It is quite possible that, owing to the number of active molecules that have managed to penetrate the cell, various levels of modifications can be observed, such as those Dr. Braun has described.

Dr. Ball: The following question is of particular interest to the morphologist: Is this induction process a cellular phenomenon or is it one of tissues? Two types of experiments are very pertinent in this regard. If one grafts the genetic tumors, described by Dr. Kehr, into a host tissue, the host tissue does not become a new tumor. On the other hand, as de Ropp showed, if one grafts a crown-gall tumor, even one that has grown a long time in sterile culture, into a host plant of susceptible variety, new tumors are produced at the point of juncture of normal and tumor tissue. de Ropp presented evidence that the new tumor was a composite of normal (stock) and tumor (scion) tissues. We should probably ask Dr. Riker if he could give us some idea on whether this matter is a single cell phenomenon or one of many cells. Another point of interest to me is the question of whether the crown-gall tissue gains new enzyme systems by its induction or is this merely an indication of enhanced activity of enzymes already present? In this regard, we can give some tentative conclusions obtained by working on the hexokinases of crown gall of *Vinca*, which Dr. Braun was kind enough to send a year or so ago. We have found that the hexokinases in this crown-gall tissue are the same as in normal stem tissue of the plant, yet their activity in the crown-gall tissue is several times that of the normal tissue.

Dr. Riker: I would like to discuss this question, but several questions have been raised about Dr. Braun's paper, and he should have first chance at rebuttal.

Dr. Braun: One interesting aspect of the crown-gall story, as it is now unfolding, is concerned with the explanation of the continued abnormal and essentially unregulated proliferation of the crown-gall tumor cell in terms of the permanent unblocking of a series of growth-substance synthesizing systems. These systems are precisely regulated in normal plant cells. The problem confronting us now is to determine how this unblocking is accomplished by the tumor-inducing principle. Professor Gautheret has pointed out the importance of characterizing the tumor-inducing principle for the ultimate solution of this problem. I think most of us who are actively working in this field are in accord with this viewpoint. Yet there is no assurance, even if we now knew precisely what this factor is, that we would be any closer than we are now to an understanding of its mode of action in converting a normal cell to a tumor cell. There are, as we know, many different agencies of the most diverse types, that are capable of transforming normal cells to tumor cells. Among these might be mentioned radiant energy, hereditary factors, a host of carcinogenic chemicals, and viruses. Some of these have been very precisely characterized, yet we know little or nothing concerning their mode of action in accomplishing the cellular alteration. We have, therefore, concerned ourselves primarily with the question of what the tumor-inducing principle does, rather than what it is, in the hope of gaining an understanding of the nature of the autonomous growth of cells.

Dr. Skoog: One point in the discussion of the cell-division factor in crown-gall extract needs to be clarified. It may have been implied, or you may think, that the factor is characteristic of the crown-gall cells only. Actually, cell-division factors can be obtained, I think just as well, from normal tissue extracts. Furthermore,

there is much more cell-division factor in normal plant tissues than might appear, because there also are other factors that mask its activity.

Dr. White: Dr. Ball raised one point which I think needs clarification. I am not sure whether it is a matter of misunderstanding or not. In my own experience, at least, if one grafts bacteria-free crown-gall tissue, one never gets new tumors at a distance from the site of implantation. If that is what Dr. Ball means by secondary tumors, then I think either he has had different experience from ours, or he has misunderstood the facts. If, however, by secondary tumors, he means tumors that are not the direct result of the bacteria, then, of course, those arising at the site of implantation *are* secondary tumors. I think this needs clarification. You get a primary tumor at the site of inoculation of bacteria. You get a secondary tumor that arises at a distance and it is sterile. You graft tissue that has been derived from the secondary tumor and cultivated in tissue culture back into a normal plant. And you do get a massive tumor at the site of implantation of that tissue. But in my experience, you do not get metastasis from such a graft. Perhaps others have had other experience. If I found a secondary tumor arising at a distance from such a graft, I would suspect that I had bacteria in the culture in which they did not belong.

Dr. Thimann: Dr. Braun's last query about the conversion of a pith cell into a tumor cell could be directed toward an earlier stage, namely, how did it become a pith cell in the first place? If there is a direct relationship between genes and enzymes, then the genetic material of the plant must confer on it enzyme systems capable of producing an auxin and a kinetin, because these would be presumably required in normal growth. In what has been called the "differentiation" of a pith cell, at least one of these factors must have been lost.

These thoughts also raise some other considerations. Although the terms "cell-division factor" and "cell-enlargement factor" have been frequently used, it probably would be generally agreed that they are only terms of convenience. Auxin, it is true, causes enlargement of some cells, but as Dr. Morel points out, it causes cell division in artichoke and in cambium. Kinetin causes cell division in several tissues, though apparently only in combination with auxin; but in leaf tissues, kinetin clearly causes cell enlargement. Not only are these terms somewhat unsatisfactory but they raise the general question of what is meant by the expression "growth by cell division." It is obvious that if cells only divided, they could not become any larger and therefore could not have grown. Growth therefore, necessarily, includes cell enlargement. But when cells divide, it is possible for them to go on enlarging for a longer time and one of the problems of growth somehow resides in the fact that if cells are made to enlarge, as by auxin treatment, this is a finite process. They may reach 5 or 10 times their initial volume but that is about the limit. However, as soon as they have divided they can enlarge some more. Thus what is required is neither a "division factor" nor an "enlargement factor" but something that will enable the process to continue indefinitely.

Tissue Culture in the Study of Animal and Human Tumors [1,2]

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Cancer has long been a very proper and popular subject of study by tissue-culture methods. In the bibliography of tissue culture (*1*), since the early 1920's, there has been a rather stabilized output of published communications on this subject with relatively slight shifts in emphasis. At the present time, and through the past decade, we find some acceleration of this pace and a more thorough exploration of some of the basic areas, which I think gives you an idea of what this section will include. This, of course, parallels the same sort of trend that appears in other branches of tissue-culture work, and I would like to mention a few factors that probably relate to this: (*a*) the development of new microscopic techniques, particularly phase and electron microscopy, which are particularly useful for cultivated cells; (*b*) attainment of mass culture methods adapted for various uses, including actual measurement of growth, *e.g.*, counting of cells while they are still viable; (*c*) development of media that are largely or entirely synthetic so that studies of metabolism begin to have meaning and replace the older studies of "growth promotion," etc. Finally, and I think very importantly, the influence of the Conference at Hershey, Pennsylvania, 10 years ago, the *esprit de corps* of the ensuing association, the recurring summer courses bringing people of many interests into common endeavors, and, in fact, a general socializing of what was formerly sometimes too esoteric and often without the feeling of a proper discipline—all this is a very remarkable phenomenon of the past few years.

In reviewing the ledger of our accomplishments and failures in cancer research, we should perhaps consider how we might use tissue-culture methods in the study of cancer. I have tried to list them in the following categories:

1) We may use these methods in elucidating the differences which distinguish tumor cells from other animal cells: Here we must remember that cancer is defined in terms of its "malignant" interaction with the tissues of the host, so there may not even be an answer to this question

[1] Received for publication May 28, 1957.
[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8–12, 1956.

which I raise. Yet most of us intuitively have a strong belief that malignant behavior has its counterpart in some definite peculiarity on the cell level, such as biochemical, morphological, immunological, or what you will.

One thing has made this row a hard one to hoe; that is, of course, the inductive nature of the reasoning and the obvious restriction that, since we are looking for a critical difference or peculiarity, a single exception which turns up can disprove the rule; and this has happened. The situation was even worse when homologous normal-malignant tissue pairs were hard to come by. Now that malignant transformations are a commonplace, we may have autologous pairs of common origin, and there is one precaution in regard to this which I will mention.

a) *Growth rate as a criterion of distinction.*—This is something that investigators have been inclined to study in relation to malignant growth, although few tumors, if any, proliferate as rapidly *in vivo* as intestinal crypt cells or, for that matter, as the mature liver when given cause to regenerate. Since the essence of tissue-culture technique has generally been to procure a maximum growth rate, in whatever is cultivated, the method is probably better suited to detect those differences between tissues which have nothing to do with growth rate. The high mitotic index sometimes observed in tumor cultures, for instance, may largely reflect a slower progression of the somewhat impaired mitotic figure. Perhaps, also, as was suggested in a classical study by Fischer (*2*), frequent death of cells after division results in a higher mitotic index than the eventual growth would account for. Where cell strains that have undergone malignant transformation grow more rapidly than before, the transformation might be explained by competitive survival of a cell type that was slightly more rapid in growth rate. As between 2 cell lines with 20- and 30-hour intermitotic times, for example, the latter would virtually disappear in 2 or 3 weeks in a mixed culture. It would be important to look for a reverse transformation (from malignant to nonmalignant) with further increased growth rate. If the disappearance of malignancy in cultivated malignant cells is a sufficiently common event, this might be seen. On the other hand, if transformation is not due to a single cell mutation but rather to the change-over of an entire culture, we might even see sometimes a retardation of growth along with the change. I trust some of the discussers may be able to cite instances in this regard.

b) *Morphological distinctions.*—Most of us can tell a tumor cell from an undifferentiated normal cell in culture with fair reliability. I believe we can recognize the particular tumors with which we work and can distinguish them from the others. Perhaps the best criterion is one of degree. One thing that has received a good deal of attention is the variability of tumor cells in nuclear, nucleolar, and cell size and configuration and the high frequency of unequal mitoses. I am not certain that this morphological variability has been shown to be a function of degree of malignancy. The so-called normal cell may, under abnormal conditions of cultivation, show these same phenomena. Certainly, as a rough

generalization, one has the impression that the tumor cell has forgotten how to segregate its chromatin. I am not sure whether one should call pinocytosis a morphological or a physiological phenomenon. Insofar as it characterizes tumor cells against most nonmalignant cell types, pinocytosis has important physiological implications. We think of the normal cell as being impermeable to many molecules. For example, some unpublished work that Miss Sage and I did a few years ago (*3*) showed that embryo fibroblasts were quite impermeable to P^{32}-labeled nucleotides, although these materials are growth promoting; the molecules must be broken down in order to be absorbed through the cell, yet in motion pictures we see some other cell types, especially malignant ones, ingesting large quantities of water along with solutes and suspended particles, and, undoubtedly in this case, these molecules, which don't get into quiet cells, must gain entrance to those showing pinocytosis. It would be of interest to know, for example, how long it takes one of these cells to ingest its own volume of water and what variety of things get into a cell only in that way.

c) *Cell physiology.*—Tissue cultures are well adapted to the study of cell physiology and, indeed, may represent the only way in which it can be directly approached. In regard to the fowl tumor, perhaps people have shied away from its use in such matters as comparative cell physiology because they have the feeling that they may be deluded about the origin of human tumors by studying these tumors. When, however, you have a preparation that can be sprinkled into a culture of normal cells to produce cells that act like malignant cells in the body, I think the method if handled properly can perhaps be useful in comparative studies.

d) *Biochemistry.*—This has two aspects in relation to tissue culture: nutrition, in which we have much definite information; and intracellular metabolic processes, which are now generally studied by quicker means. Tissue culture has certain potentialities for comparative studies of tissues that are actually growing at equivalent rates, one normal and the other malignant, while under observation. It is also possible to examine individual cells through tracer techniques, which was done first by Pelc and Howard (*4*); and, with the use of tritium labeled compounds, it is now possible, because of the low energy of that isotope, to bring autoradiography almost down to the limit of resolution of the light microscope.

2) Another important branch of work supplements that of pathology, whose classical role has been to give final diagnoses. Tissue culture has been of signal value in supplementing the work of those whose tissues are usually fixed and promises to make important contributions to the development of cytological means of tumor diagnosis.

3) Concerning the elucidation of growth factors, a distinction should be made between nutritional requirements on the one hand and factors that may influence growth even in the presence of adequate nutrition.

Since, to all intents and purposes, the best tissue cultures are those growing under optimal nutrition, the best we can expect to do is to demonstrate the existence of inhibitors that keep optimal growth within bounds

even under conditions of good nourishment, if that, perhaps, is what goes on in the resting cells of the animal. Such an inhibitor might be specific for the tumor where it could be of therapeutic value. We might find agents that inhibit normal tissues but have no such effect on malignant tumors and that might be considered as physiological growth-controlling agents. We have observed agents of this sort: the aliphatic amines (*5*), low concentrations of sodium in the medium (*6*), and some ill-defined proteins or lipoproteins from liver (*7*). This finding, namely, the apparent specificity for normal tissues against tumors, needs, because of the inductive nature of this type of investigation, verification with a considerably larger variety of tissues than those studied. At the same time, we should see whether the growth inhibition is a process by itself or whether it is related to some such process as differentiation (which needs further definition for our purposes). In certain tumors, for example, the distinction between the processes of growth and differentiation is seen *in vivo* and, I suspect, also *in vitro*. The Jackson embryoma (*8*) is an example in which half the cells mature, while the tumor continues to be a malignant tumor. The factor that promotes multiplication of plant cells without apparently contributing to nutrition, kinetin (*9*), which I believe has been discussed here more than once, has not yet been shown to play a definitive role in animal tissues, and I would be delighted to have a verification or denial of that documented by someone.

4) A malignant tumor defines itself in ecological terms, that is, in terms of its interaction with tissues of the host animal. Because of our present inability to demonstrate a singular difference between normal cells as a whole and malignant cells as a whole, can we study the problem on the basis of the process of interaction? Autonomy is accomplished, of course, the moment a pure culture of cells is grown by itself, and then invasiveness cannot be demonstrated except in terms of the medium, which cells, in general, invade. Tissue culture is beginning to fill this gap through observations on interactions on the simplest possible levels. In other words, it is possible to put just a few of the components together and to see what happens, for example, the demonstration of better growth of tumors near fibroblasts by Leighton (*10*), and the reciprocal of this by Ludford (*11*). Experiments like this are very important because they may afford us the opportunity to sort out several such factors as the ability of the less viable cells to nourish those that survive longer. This can even be shown in homogeneous cultures (*12*) and it must be even more important in mixed cultures. A fairly striking example of the influence of the variable environment or of interaction is the fact that ascites tumors are especially difficult to grow under tissue-culture conditions. It would seem as if the extreme poverty of their environment has become more or less obligatory, yet in terms of growth rate the ascites tumors can hardly be paralleled.

5) Tissue culture has become useful in the examination of agents that might be of therapeutic value against cancer. Here again, we are bedeviled by the almost infinite variety of tumors and the considerable

variety of normal tissues. Such agents are in general of two sorts, mitotic inhibitors and antimetabolites. The mitotic inhibitor, to be effective, must obviously have particular tissue specificity; for one of the most effective mitotic inhibitors, namely, total-body irradiation, destroys the host through inhibition of the self-replenishing capacity of certain of the normal tissues, intestine and bone marrow (*13*) before it has an appreciable effect on tumors, except for the most radiosensitive ones. Nevertheless, mitotic inhibition, occurring as it does in many forms and through many mechanisms, is well worth further investigation.

In regard to antimetabolites, it is probably quite sensible that we should study them in general and not just on the basis of already demonstrated biochemical or nutritional findings, for I think one category of investigation may feed the other and *vice versa*. Other agents may demonstrate differential responses on the part of "normal" or tumor cells. Thus tumor cells have been found, in a simple area of study, less resistant to osmotic pressure changes but more resistant to variations in *p*H (*14*). Such studies as these have generally been carried out in a limited sense and deserve to be expanded in the direction of a wider spectrum of normal and malignant cells.

One of the earliest examples of the application of tissue culture to the treatment of cancer arose through the demonstration by Fischer (*15*), a long time ago, that increased oxygen tension was more harmful to malignant tissues in culture than to normal ones. As a consequence of this observation, many patients were treated by inhalation of oxygen in high concentration without remarkably successful results (*16*). The best results were obtained when X irradiation was combined with the oxygen treatment. This agrees with our present knowledge of the way in which oxygen reinforces radiation effects.

Along with studies on the therapy of tumors *in vitro*, it is important to study, in detail, the pathology of the sick cells. We should not be satisfied with observing "rounding," "granularity," or "disintegration" of cells. A knowledge of human pathology is necessary as a foundation of toxicology. Gey's electronmicrograph of a virus-filled cell in a degenerating culture (*17*) is an example of what can be learned in this respect.

6) In regard to carcinogenesis *in vitro*, the significant observation is not that carcinogens can be operative *in vitro* but that malignant changes occur in a manner that we might call spontaneous. Whether artificial conditions of cell cultivation or prolonged forced growth are necessary and whether the phenomenon can be attributed to simple mutation followed by competitive growth or by something more complex are among the questions of current interest.

I would like to summarize by recounting what seems most worthy of more current attention—since in the present state of promising "breakthroughs" in certain areas, we must avoid rushing to the same side of the ship. I think we need, and can readily gain, more knowledge of the basic physiology and pathology of the isolated cell and of physically and chemically defined circumstances that inhibit growth in a "normal"

fashion. Finally, tissue culture like cancer biology needs to become more a part of a general education in the life sciences. Many graduate and medical students never, I suspect, see a film such as we have seen here. This lack of specific knowledge results in the perpetuation of certain misconceptions by those not in the inner circle—of the cell as a rigid semipermeable compartment, or of the mitochondrion as a sort of egg crate.

In any event, if we might have said 10 years ago that tissue culture was a trivial aspect of cancer research, this is something we certainly cannot say today.

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DISCUSSION

Dr. Lasnitzki: Dr. Brues has admirably opened up and discussed the many problems confronting us in the elucidation of tumor-cell behavior which can be tackled by means of tissue culture. The malignant cell, however, does not start life as such, and we have been interested in studying its transition from the normal to the malignant or precancerous state, by the organ-culture technique, in tissues grown in the presence of carcinogenic agents.

As examples I would like to call your attention to the changes induced by benzo-[*a*]pyrene (3,4-benzpyrene) and by tobacco condensate in the human fetal lung—this latter experiment has been started in collaboration with Professor Sir Ernest Kennaway—and by 3-methylcholanthrene (20-methylcholanthrene) in the mouse prostate gland.

All three substances induce mitotic stimulation leading to hyperplasia of the epithelial structures. Irregular increase in cell size, multinucleate cells, and polyploid divisions were frequently observed in hyperplastic areas. Benzo[*a*]pyrene and 3-methylcholanthrene at the same time inhibit the growth of the connective tissue, while the tobacco condensate does not greatly influence it.

Figure 1, plate 70, shows a section of a human fetal lung grown *in vitro* for 3 weeks in normal medium. It consists of bronchioli embedded in cellular connective tissue. Figure 2, plate 70, is a similar culture, treated for 3 weeks with 5 μg. of benzo[*a*]pyrene per ml. of medium, showing a number of hyperplastic bronchioli, some of them occluded. During the early stages of hyperplasia the epithelium consists of small crowded cells of the basal-cell type lined by a layer of secretory epithelium. Later, irregular increase in cell size and abnormal mitotic figures are observed while the secretory epithelium degenerates and is shed.

In contrast to benzo[*a*]pyrene, which affects the bronchioli independently of size, tobacco condensate induces hyperplasia mainly in the larger bronchioli. Figure 3, plate 70, shows marked basal-cell hyperplasia with abundance of mitosis in an explant treated for 2 weeks with 500 μg. per ml. of medium. We find that the function is not impaired in hyperplastic areas but that secretion and cilia formation are increased compared with normal structures.

Prostate glands from young or adult mice can be grown in culture for indefinite periods. Figure 4, plate 70, shows a section through a normal gland from a 3-month-old mouse grown *in vitro* for 3 weeks. It consists of alveoli lined with one row of cuboidal or columnar secretory epithelium surrounded by strands of fibromuscular stroma. Experimental cultures were treated for 10 days with a concentration of 2 to 4 μg. per ml. of 3-methylcholanthrene and then carried on for an additional 10 days in normal medium. They show extensive hyperplasia in most alveoli while the stroma is completely absent (fig. 5, plate 70).

The mitotic stimulation due to the carcinogen was estimated by comparing mitotic counts in normal and in treated cultures. After 5 days, mitosis is 3 to 4 times that of the controls and remains so up to the 10th day. It then falls to the control level following the withdrawal of the carcinogen. Between the 14th and 20th day, it rises again to 4 to 5 times the control value, although the cultures are kept in normal medium, possibly due to an increased growth potential of the altered epithelium.

Dr. Pelc, of the M. R. C. Unit at Hammersmith, and I tried to correlate the morphological changes with changes in deoxyribonucleic acid (DNA) synthesis by means of autoradiography. The uptake of 8 C^{14}-adenine was assessed by determining the percentage of labeled nuclei in the alveoli of treated and untreated cultures.

We found that the uptake of the tracer was slightly increased after 7–10 days' treatment with methylcholanthrene. The increase in DNA synthesis followed rather than preceded the mitotic stimulation.

Finally, I should like to describe briefly the influence of excess vitamin A and estrone on the hyperplastic changes in the prostate. In this organ, the hyperplastic epithelium usually undergoes squamous metaplasia and becomes stratified into layers of basal and squamous cells. The secretory epithelium degenerates and is shed and replaced by flat cornifying elements.

Addition of excess vitamin A simultaneously with methylcholanthrene suppresses the squamous changes and preserves the secretory epithelium. Addition of the vitamin after withdrawal of the carcinogen also inhibits the squamous metaplasia and brings about a partial reversal of the carcinogen effect by reducing the incidence and extent of hyperplasia (fig. 6, plate 70).

Estrone added to the medium after withdrawal of the carcinogen also reduces the hyperplasia in the prostate by an increase in cornification, which causes a breakdown and shedding of the hyperplastic epithelium.

These few examples show that organ cultures are eminently suitable to follow changes induced by carcinogens in differentiated tissues as opposed to studies on a purely cellular level. We find that the carcinogens directly induce hyperplasia and often metaplasia, which may be considered initial stages in the process of carcinogenesis and which, in turn, can be modified by such factors as excess vitamin A or hormones.

PLATE 70

Cultures of Human Fetal Lung

FIGURE 1.—Control explant after 3 weeks' growth *in vitro*, showing branching bronchioli embedded in cellular connective tissue. Hematoxylin and eosin. × 110

FIGURE 2.—Similar culture treated for 3 weeks with 5 μg. per ml. of benzo[*a*]pyrene. *Note* hyperplastic bronchioli. Hematoxylin and eosin. × 280

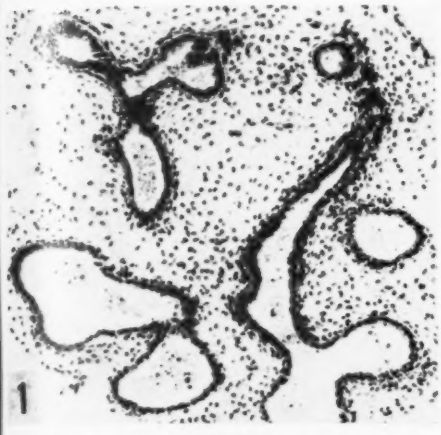
FIGURE 3.—Culture treated for 2 weeks with 500 μg. per ml. of tobacco condensate showing hyperplasia of larger bronchioli. Periodic acid-Schiff method. × 110

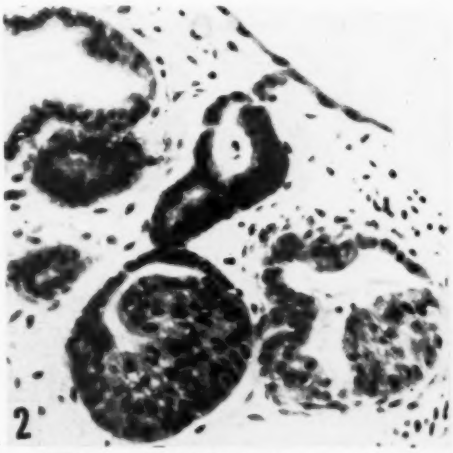
Cultures of Mouse Prostate Glands

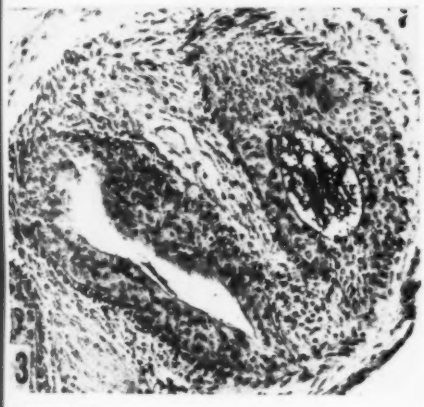
FIGURE 4.—Control culture after 3 weeks' growth *in vitro*, showing alveoli surrounded by fibromuscular stroma. Hematoxylin and eosin. × 100

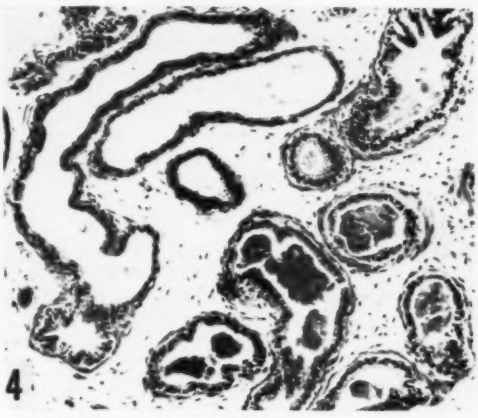
FIGURE 5.—Culture treated for 10 days with 4 μg. per ml. of 3-methylcholanthrene and carried on a further 10 days in normal medium. *Note* extensive hyperplasia and stratification in alveoli and absence of stroma. Hematoxylin and eosin. × 115

FIGURE 6.—Culture treated for 11 days with the same dose of 3-methylcholanthrene and carried on for 9 days with 1500 I. U. of vitamin A per 100 ml., showing partial reversal of hyperplasia. Hematoxylin and eosin. × 80









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Dr. Earle: I want to bring to your attention certain data from a series of researches that have been in progress in our laboratory by a team of workers headed by Dr. Katherine Sanford. Years ago, we tried to change the normal mouse fibroblast in tissue culture to a cell type able to give rise to sarcoma when reinjected into mice of the same strain from which the cells arose. We did effect a change-over of the cells, but suddenly the problem boomeranged. It is now no longer a problem of changing the normal to the malignant cell in tissue culture; the problem that now has to be solved is whether there is any way to keep the cells from a change-over.

Approximately 5 years ago, a series of cultures was started from an 87-day-old mouse, strain C3H/He. The tissue was taken from the adipose and subcutaneous tissue of the flank area and the cultures were planted in a stock culture medium of 40 percent horse serum, 20 percent 1 : 1 chick-embryo extract, and, the rest, a balanced saline solution. All cultures since that time have been maintained in this culture medium. A number of early injections was made from the tissue cultures into mice of the same inbred strain from which the cells were taken; none of them showed any evidence of giving rise to tumors at the injection sites. When the strain of cells had been cultured about 3 months, a single cell was isolated from it and a clone strain of cells was originated from this single cell. After 1 of the cultures had been grown to a large size, it was randomly cut up to give 8 fragments; these were explanted and each was considered the origin of a separate substrain of cells. Each substrain was further divided into 2 lines of cells—1 on cellophane substrate and 1 on chicken-plasma clot, all in Carrel D-3.5 culture flasks. These 8 substrains were run on for over a year. At intervals during that time, cultures of each of the lines were injected. Gradually line after line began to develop the ability to give rise to sarcoma at the site of injection. You can run just so many cultures in a laboratory, and, of these 8 substrains, we had to take the cultures of 14 of the lines out behind the woodshed and shoot them. We kept cultures of only 2 lines; both had been on cellophane substrate. The 2 remaining lines are really the subject of our discussion today. We will arbitrarily label them the high line and the low line.

When these 2 lines had been carried for some time in tissue culture, we became aware that the cells of the 2 lines were morphologically different from each other. Figures 1 and 2, plate 71, show the morphologies that developed in the 2 lines that originated from the 1 single isolated cell. Figure 1 shows the typical morphology of cells of the low line; figure 2 shows cells of the high line, which look like normal fibroblasts for all purposes.

When the cultures of the high line were injected after 1½ to 2 years of life *in vitro*, they had the ability to give rise to sarcomas in approximately 97 percent of the mice injected. Cells of the low line at that time were able to give rise to sarcomas in only 1 percent of the mice injected. More than 5 years after the original cloning, the high line is still able to give rise to sarcomas in over 60 percent of mice into which its cells are injected. The low line is still jogging along at less than 10 percent even when extremely large inocula are used. That is a spectacular difference. Remember, those 2 cell lines arose from 1 single cell and both have been maintained in the same medium. Probably the most extraordinary point, however, is that, in spite of their widely different abilities to give rise to sarcoma on injection, their rate of proliferation in the same medium in tissue culture is identical.

If the tissue of these tumors was identical with the strain of mice from which it arose, neither one should be able to serve as an antigen to induce immunity, which would block the growth of a later injection of cells. This is not the case. Either strain, when injected in small dosage, acts as an antigen inducing an immune reaction in the host, which completely blocks the development of tumors from cells of either line subsequently injected. Also if we use irradiated animals, the low line becomes able to develop sarcoma in approximately 50 percent of its injections.

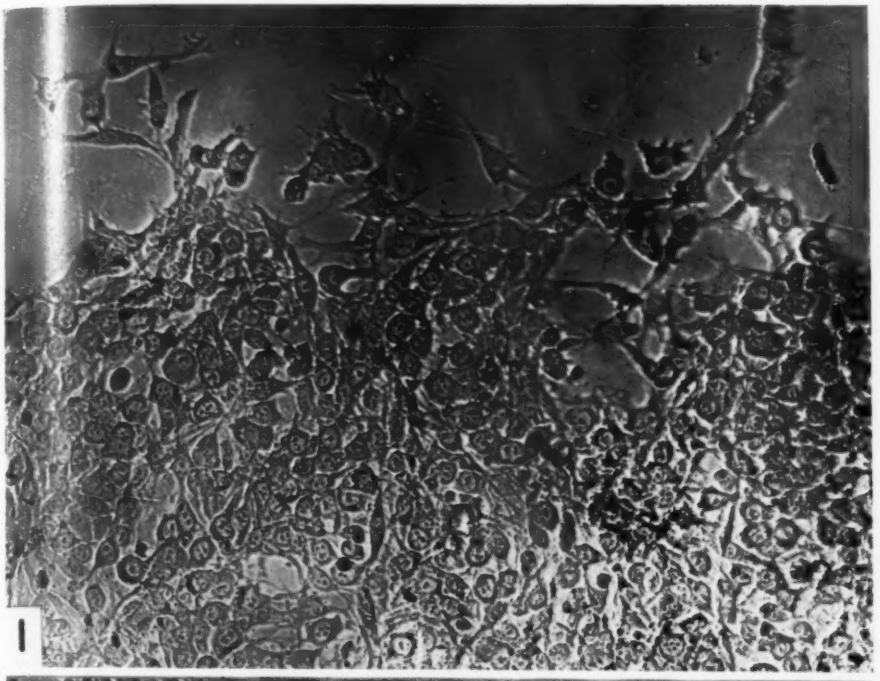
Dr. Mark Woods and Dr. Dean Burk examined the metabolism of these 2 lines for us, and they report that the metabolism of the high line is tremendously anaerobic, and as anaerobic as the most malignant cells they have ever studied; the metabolism

PLATE 71

Fibroblasts derived from a single-cell isolation from a C3H/He mouse which have segregated into 2 lines of different morphology and pathogenicity.

FIGURE 1.—"Low line"—epithelial-like, of low tumor-producing capacity.

FIGURE 2.—"High line"—fibroblast-like, of high tumor producing capacity.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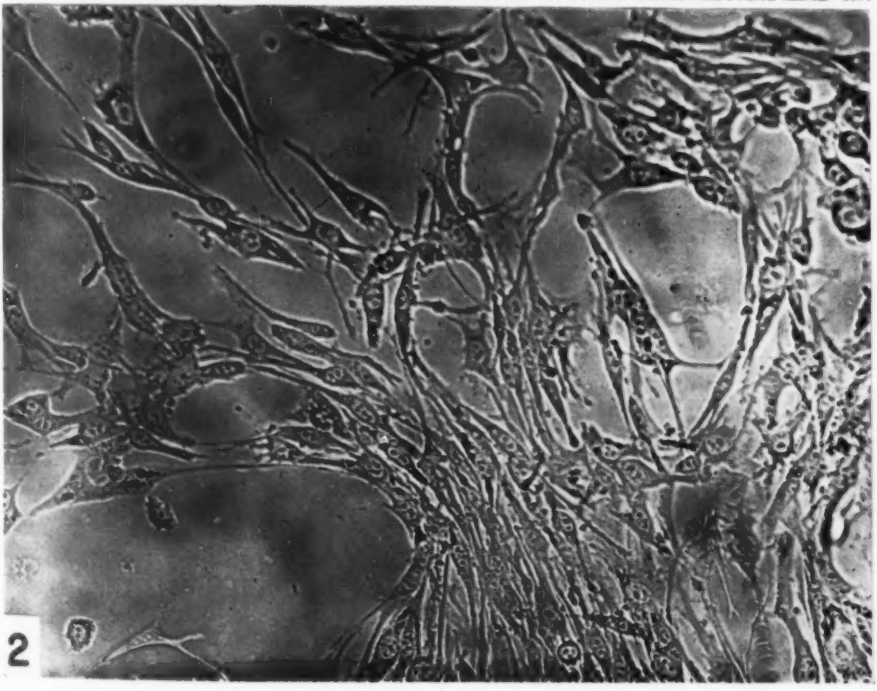
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of the low line is much closer to that of a normal cell or a cell less altered from the normal.

Sarcomas grown from the low line in irradiated mice were cultured and their cells studied in culture for an extended period. All of these cultures had acquired a high capacity to produce sarcoma on reinjection. Gradually, after the first 6 months in culture, their sarcoma-producing capacity decreased, becoming again similar to that of the original low line.

When we injected mice of the same strain and examined sections through the injection sites at different intervals of time, we found that cells of the high line became rapidly established at a high rate of cell proliferation, with little or no necrosis; they aroused a tremendously vascular reaction from the host; the cells were entirely at home and ultimately and inevitably killed the animal. The low line, as early as 24 to 72 hours after injection, showed an extremely low rate of mitosis and was unable to induce a strong vascular response from the host; their growth was very erratic and unsatisfactory. I shall not try to interpret these data at this time.

Dr. Gey: Dr. Brues has mentioned a few things that we had hoped to present in a more elaborate form. I shall try to confine my comments to our observations on some of the comparative responses of normal and malignant cells in continuous culture. Beginning with the primary explantation, we have found as others have, that some normal and some tumor cells do not grow well at all after varying periods in culture in a variety of media and soon peter out. Why this is, is a matter for consideration elsewhere. There are some cell types, however, that, once isolated, proliferate well in continuous culture. Many normal and malignant cells belong in this category. Some of these may show good stability; others, may, after a period of cultivation, change into a variety of altered normal cells and even into malignant ones. There are good examples of them in our work and in that of others. Dr. Brues has referred to the need for the isolation of some autologous strains for the comparative study of normal and malignant cells. We have made an effort to isolate such strains from animals and man and have succeeded to some extent.

With one set of isologous strains, we are now able to emphasize some important differences between the normal prototype strain and the derivative malignant strain. I refer particularly to some of the differences that we have observed in the normal strain "14p" of rat fibroblasts, isolated in 1938, when compared with the over-all picture seen in its tumor derivative, strain T-333. Strain 14p differs from the tumorous strain T-333 in a number of ways. The stable normal strain still shows a growth rate of approximately one fifth the rate of growth of its tumorous derivative. It has a relatively low mitotic index, roughly one fifth of the malignant derivative. The normal strain shows some degree of ploidy but this varies somewhat from culture to culture. Until more careful studies are carried out, we cannot be certain whether the examples of irregular polyploidy (aneuploidy) in this normal rat strain are as common as those that have been observed in many of the so-called normal strains isolated from human fetuses and from adult tissues. In the tumorous derivative T-333, we see much irregular polyploidy (aneuploidy). In fact, in some of the strains related to these of isologous origin, we have seen multinucleated cells with nuclei of different sizes produce multinucleated daughter cells. Interrupted telophases are also sometimes found in some of the daughter cells which appear to escape the refusion reaction that more commonly occurs where the telophase bridge finally pulls the two major daughter-cell masses together in a kind of endoreduplication. We may speak of this and other types of abnormal mitotic behavior as an aspect of malignancy, yet much time and effort will be required to unravel this complex mitotic display and judge its full meaning. A careful isolation of these irregular polyploids and of other abnormal members within the normal cell strain may eventually permit us to reveal the potentials of these cells as different from other members of the population that may be restraining them. There are, of course, other factors that appear to be important in making comparisons between normal and malignant cells of isologous origin. Cell cohesion has been referred to as perhaps important in determining some

aspects of the metastatic spread of cells. The normal prototype cells under discussion, strain 14p, show good cellular cohesion, while the malignant derivative, strain T-333, exhibits poor cohesion as we see them in some of our continuous cultures. The normal cohesion would appear to be related to the ability of these normal cells to produce numerous microfibrils, whereas the tumor cells produce fewer. The normal cell displays long and fairly stable mitochondria, whereas the malignant derivative usually shows fewer, which tend to be shorter and often beaded. The mitochondrial display may vary somewhat from cell to cell in the same culture of many strains. The differences noted between strains 14p and T-333 are significant.

In regard to the appetite that these cells manifest in the form of pinocytosis, the normal prototype shows very little compared with the great pinocytotic activity found in its malignant derivative. Under abnormal conditions of cultivation, one may vary the rate of this activity in each of the strains, but it will not change the major differences that we have observed. Concomitant with the exaggerated pinocytosis found in the tumorous strain T-333, there occurs an enlargement of the juxtanuclear area or centrosphere. An enlarged centrosphere is, of course, found in other cells, both normal and malignant, yet I do not know of any specific comparisons of isologous lines covering this point. Other differences in the responses of the normal prototype and the malignant derivative should be brought to your attention as important aspects of their behavior and some may be related to the above incorporation phenomena. If we challenge the normal prototype 14p with the virus of Eastern equine encephalomyelitis, it may be shown to be solidly resistant to moderate doses of virus. When high doses of virus are added, a few cells degenerate and only minimal virus may be produced. By comparison, the malignant derivative is found to be very susceptible, demonstrating in tissue culture a kind of *preferential oncolytic effect.* In some of our efforts to sterilize the culture medium by irradiation with fast electrons at doses ranging from 2 to 5 million rep (roentgens equivalent physical) we obtain a brownish discolored medium. In this medium our normal prototype cell shows a very poor growth response, yet its malignant derivative shows good growth in this greatly altered irradiated medium. It would be interesting to determine the nutritional differences between the irradiated and the unirradiated medium in relation to the requirements of these two isologously related cell strains.

Some aspects of the metabolic pattern of these two strains may be of interest. The tumor derivative shows a higher level of metabolic activity, judged from their comparative growth rates and other aspects. The aerobic glycolytic activity of the normal strain is about 50 percent of the tumorous strain. The anaerobic lactate production is only one sixth that of the tumorous derivative. The respiratory rate of the normal strain is about two thirds that of its derivative. We must keep in mind that these figures relate to responses in tissue culture and that the normal tissue usually may be even less active *in vivo.*

In conclusion, may I refer briefly to our evaluations of some human cells in an effort to establish isologous normal and malignant strains. In the case of the HeLa malignant-cell isolation we were able to maintain its normal prototype strain for almost 2 years, yet it consistently grew only about one twentieth as rapidly as its malignant derivative. The normal prototype of HeLa produced what appeared to be keratinized cells and eventually its cells differentiated out of proportion to the rate of new cell growth. Human strains of isologous origin are needed if we are to establish some fundamental differences in the responses of the normal as compared with the malignant derivatives in man. We have been hearing from Dr. Moore and others about alterations in some normal human strains following their establishment in continuous culture. We have also learned that some of these may grow as homografts in suitable tumorous human hosts. This response, however, would appear to be different from that produced when a rapidly proliferating autonomous tumor-cell line is inoculated as a homograft. I believe these findings emphasize again the importance of working with isologous strains.

Dr. Biesele: Dr. Brues has mentioned the use of tissue culture in the study of cellular responses to mitotic poisons, antimetabolites, and the like. I am particularly interested

in the effects of such agents on tumor cells in tissue culture. Our hope has been that a study of differential effects on neoplastic cells might make some contribution to the chemotherapy of cancer. In this Conference we have heard a great deal about adaptability of cells in tissue culture to environmental changes and, particularly, to changes in the nutrient medium. We have also heard about changes in response to viruses through prolonged cultivation, and about alterations in other respects. Hence, adaptation in tissue cultures might be expected to produce difficulties in the testing of chemicals or, rather, in carrying over the results of such tests to the cancerous animal or man. This would be so especially with cell strains that have been cultivated for a long time *in vitro* and away from the regulatory effects of the host animal. Dr. Earle's remarks are particularly pertinent here.

One should therefore not be surprised that Eagle and Foley (*1*) in their initial testing of semisynthetic media in which cell strains of normal and of neoplastic origin had been maintained should have reported the absence of differential toxicity of a variety of agents for cell strains of neoplastic origin.

Perhaps cultivation in the same medium for some time had imposed a degree of metabolic uniformity on the different cell strains. According to Levan (*2*), some of the human cell strains of normal origin were undergoing changes in their chromosomal constitution, and Dr. Gey has mentioned this. These included changes toward the state of irregularly high ploidy with some structural rearrangements. Moore, Southam, and Sternberg (*3*) noted that implants of human epithelial cell strains of normal origin behaved just as implants of strains of neoplastic origin when put into volunteer patients or cortisonized animals.

All of this I regard as some justification for the use of fresh explants in the tissue-culture screening of chemicals for differential toxicity to cancer cells. Of course, if the test is simply to be one of toxicity, with no attention paid to differentials, changes toward neoplasia in cell strains of normal origin would be of little importance.

But the composition of the medium would certainly influence the results, especially with various antimetabolites. Azaserine, for instance, is an antagonist of glutamine, which is in high concentration in Eagle's receipt (*4*). In order to change the human epidermoid carcinoma #1 of Toolan, from the control appearance as seen in figure 1, plate 72, to that seen in figure 2, plate 72, ½ m*M* per ml. of azaserine over 1 day is required. This is a much higher concentration of azaserine than is needed to influence mouse cells adversely in a serum-embryo extract medium. Similarly, γ-glutamyl hydrazide shows little of its mitotically inhibitory effect on human tumor cells in Eagle's semisynthetic medium until the glutamine concentration is appreciably diminished.

However, if human cell strains are to be used for screening, and a test involving differential toxicity is desired, perhaps it would be possible to use recently isolated normal cell strains, preferably epithelial, the old strains of normal origin being discarded at frequent intervals.

There has been some discussion of Dr. Skoog's kinetin at this Conference, and I would like to tell you a little about our experience with kinetin on mammalian cells. Lettré and Endo (*5*) have reported no effect with kinetin on a variety of animal cells, and we have found that only relatively high concentrations of it show some inhibition of mouse sarcoma 180 cells and of the epithelial cells of mouse embryonic skin explants. A different result is seen with the riboside of kinetin. This agent, 6-furfurylamino-9-β-D-ribofuranosylpurine, is more toxic than the free purine, and especially so to fibroblasts (*6*). The effects are more striking with some human cell strains (*7*). At the 0.01 m*M* level fibroblasts are largely wiped out, while the mitotic incidence in the cancer strains is little reduced. Unfortunately, this differential sensitivity is not displayed solely by fibroblasts, for I understand from Dr. Moore that cells of Toolan's epidermoid carcinoma #3 are rather sensitive as well. This particular pattern of response is only one of a number shown by cells in tissue culture toward 6-substituted purines and their ribosides, some of which are, as you know, finding some clinical use because of their inhibitory effects on certain neoplastic cells in man.

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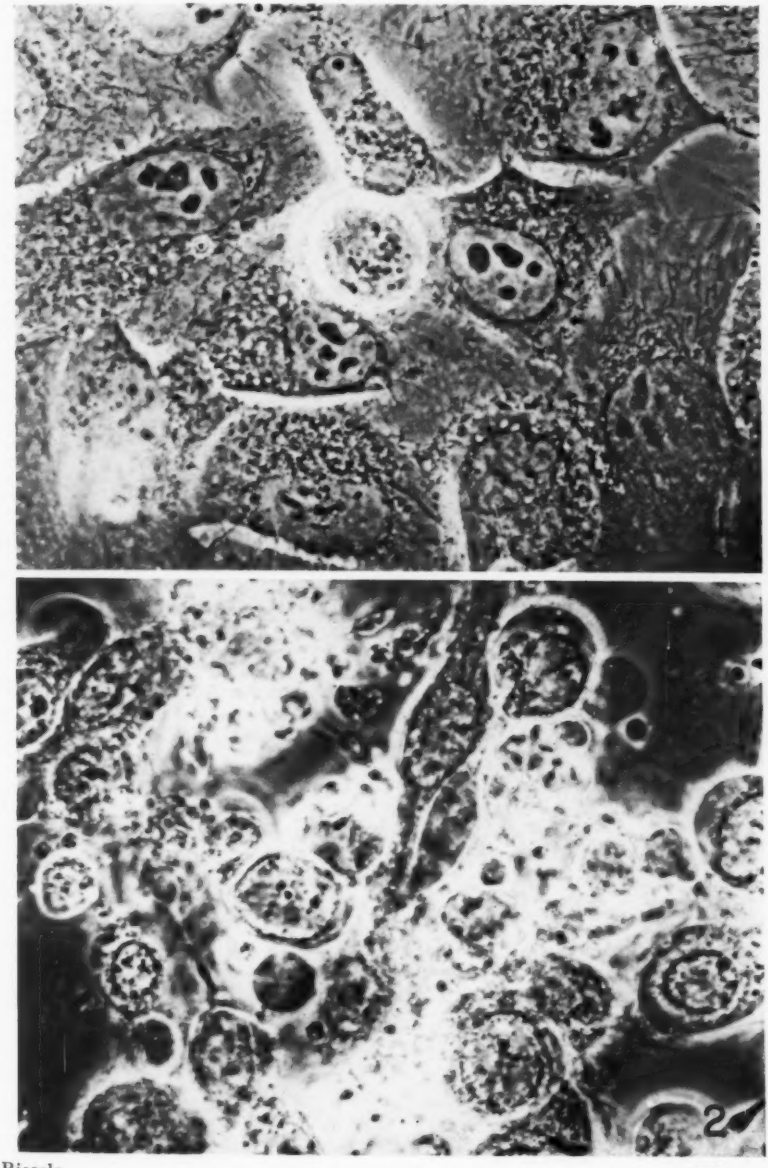
PLATE 72

FIGURE 1.—Cells of Toolan's human epidermoid carcinoma #1 in control culture on glass. Phase contrast. × 727

FIGURE 2.—Cells of human epidermoid carcinoma #1 after 24-hour exposure to 0.5 m*M* azaserine. *Note* chromatin margination and small size of nucleoli. Phase contrast. × 727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Dr. Harris: May we return to one of Dr. Brues' comments concerning the possible disappearance of malignancy in malignant cells? This certainly is an intriguing concept, and yet I wonder whether it hasn't perhaps an explanation in terms of genetic factors and incompatibility. There is a good body of evidence that for the transplantation of mouse tumors, in particular, genetic factors are crucial and that one can explain the take or the lack of take from the basis of histocompatibility genes. If one assumes that cells are carried *in vitro* for extended periods, it does not seem unreasonable to expect that changes at the histocompatibility loci could occur and, if such were the case, that an antigenicity not originally present would develop even if these were placed back in the same original strain.

Dr. Woolley: I have been struck by Dr. Earle's finding on changes that occur in the cultivation of cells and I am reminded of what happens in bacterial nutrition. It is frequently seen that an organism which, let us say, requires thiamin when cultivated for many passages will finally grow without thiamin, which it then makes for itself. If these tissue cells learn how to make, let us say, vitamin B_{12}, then they would be exactly comparable to the situation in bacterial cultures. It has been mentioned previously that some animal cancers have acquired the ability to synthesize vitamin B_{12}.

Captain Poley: I would like to introduce a device that I have used over the past 4 years, which could probably be used in tissue-culture work. I have used it quite successfully on a chromogenic strain of *Pseudomonas* in studying pigment production. A culture medium was poured onto the glass of an ordinary 13 × 13-inch picture frame and the surface uniformly inoculated with bacteria. Strips of filter paper, saturated with the various materials I wanted to study, were placed in a crosshatch manner on the surface of the medium. The strips were dried and sterilized under ultraviolet light, prior to implanting. A schematic study of vitamins, amino acids, minerals, and other chemicals was made for the purpose of biotic or antibiotic synergism or anergism. At the edges, where the strips project individually, controls of each material were obtained. At the junctions, the combined effect was obtained. This is truly a gradient plate because concentration of the materials at any particular point from the junctions of the paper strips is relative to the proximity of the strips and the rate of diffusion in the medium, so that the effect may be at the junction or at a point distant from it.

Dr. Melnick: It has been pointed out many times at this Conference, that the use of cells from single clones has great advantages for certain types of work in the field of cancer. I would like to mention some relevant experiments by Dr. McCollum and Dr. Foley in New Haven, who have developed a relatively simple procedure for isolating clones of cells of the Detroit-6 line, that may work with other cell lines also. They have shown that the feeder layer for the Detroit-6 cells can be supplied by a monolayer of human amnion cells that cannot be passed serially under conditions that can be used for passing the Detroit-6 cells. Into fully grown amnion cultures in ordinary test tubes, a dilute suspension of Detroit-6 cells is added. Most cultures receive a single cell in 0.1 ml. Once grown out, amnion cells metabolize very slowly, so that the phenol red remains alkaline (red) for some time. After the Detroit-6 cells are inoculated, in about 2 or 3 weeks, a certain number of cultures turn yellow indicating that actively metabolizing cells are present. Examination of such cultures under the microscope often reveals the presence of a colony of Detroit-6 cells nestling on the amnion monolayer. Only cultures with one colony are used for subsequent passage. The clonal cells, which are the only ones in the culture tube able to initiate new cultures, are released by mechanical means.

Dr. Gey: I think there ought to be a word of caution added to this discussion regarding the function of contaminating viruses in the cellular responses during continuous cultivation. I am reminded of some early work done, in collaboration with Dr. Earl Burky, Mrs. Gey, and myself, on the growth of the Brown-Pearce rabbit tumor in culture. When we first received this tumor from Dr. Otto Saphir, of the Nelson Morris Institute, Chicago, it was established in culture easily and maintained for a long while. In a later attempt to re-establish the growth of this tumor in tissue

culture, we failed completely, even after several tries. We were not prepared then, as we are now, to evaluate the function of background-contaminating viruses. We believe, however, the responses obtained had something to do with this hazard. The addition of a virus, experimentally, to a culture may lead to long persistence. Dr. Bang and I have found this to be true with some of our cell strains. In all these evaluations, one must keep in mind this question of the passenger agents in normal- and tumor-cell populations and their function in altering the behavior of the cultured cell strain.

Dr. Markert: Several participants during this Conference have mentioned the desirability of obtaining metabolic or biochemical criteria for identifying different cell strains in tissue culture. I think table 1 presents the sort of information that might be useful in cell identification. These data were obtained by the use of radioactive tracers in tissue culture. In column 1 of the table are listed several cell strains commonly used at a number of laboratories. These particular cultures were prepared by Dr. Merchant at the University of Michigan. By growing these cell strains in the presence of radioactive glucose and then measuring the amount of radioactivity incorporated in the amino acids of the cellular proteins, it was possible to discern characteristic patterns of metabolic activity in the different cell strains. The data are listed as ratios of the quantities of radioactivity incorporated in each amino acid compared with the amount of radioactivity in glutamic acid. The table shows that conspicuous metabolic differences distinguish some cell strains from others. For example, human embryonic lung and the Detroit-6 strains both accumulate relatively large amounts of aspartic acid compared with the other cell strains. Likewise, the accumulation of proline is notably different in different cell strains. Such data as these provide a sort of biochemical profile of a part of the metabolic activity of these cell strains and is, I think, exactly the kind of information that will enable us to draw biochemical distinctions between different cell strains in tissue culture, even when morphological criteria prove inadequate for distinguishing one cell strain from another.

TABLE 1.—*Relative radioactivity (compared to glutamate)*

Cell strains	Amino acids				
	Alanine	Aspartate	Glycine	Proline	Serine
Chang's liver	.16	.77	.12	.09	.24
Human embryonic lung	.12	2.65	.40	$<$.01	.09
Detroit-6	.24	2.0	.26	$<$.01	.45
HeLa 10	.17	.43	.22	.07	.22
HeLa 4	.14	.99	.21	.02	.15
"L" strain	.14	.58	.07	.17	.18
LLCM 1	.17	.35	.20	.03	.27

Dr. Skoog: I would like to ask Dr. Earle if there are any changes in chromosome configuration or any other cytological evidence of changes in the chromosomal mechanism in the strains with different abilities to form tumors?

Dr. Earle: As yet, there has been no detailed study of that. I may say it is our experience with these clone strains that any of them will throw occasional cells of much larger size and abnormally large chromosome number.

Dr. Murray: Dr. Lasnitzki, would you tell us something about your tobacco condensate, what it represents and what it contains?

Dr. Lasnitzki: The tobacco condensate was obtained from Denicotea filters or Lindsey's smoking machine, which smokes 12 cigarettes per hour at a temperature similar to that of human smoking. We used the neutral fractions from which nicotine and phenols had been removed in a concentration of 500 μg. per ml. of culture medium.

Dr. Bang: Dr. Earle, will you tell us whether this tumor, that now fails to be a tumor on transplantation in the mouse, does so because it grows rapidly at the start

and then degenerates or is there any quantitative data on what happens when you put it into the mouse?

Dr. Earle: Within 48 hours after injection, Dr. Sanford has found that the rapidly growing strain will proliferate rapidly; it shows a higher percentage of mitoses and little necrosis, and it is already beginning to arouse a vascular reaction from the host. The low strain is lacking in those characteristics. Since these differences were observed as early as 48 hours after injection, they do not arise from that immune reaction which implanted homologous tissue characteristically arouses at about 8 to 10 days after injection.

Dr. Federoff: Several years ago we obtained a flask of strain L cells from Dr. Earle and grew them in the medium recommended by him and his associates. Later, these cells became quite "unhappy" and we began to modify the medium. When we decreased the embryo extract, the cells grew better. We are now growing strain L cells, without embryo extract, in a medium consisting of 20 percent horse serum, 20 percent synthetic medium 1066, and 60 percent Hanks' balanced salt solution. Recently we again added embryo extract, but the cells did not grow in medium containing even such low concentrations of embryo extract as 2 to 5 percent.

Six months ago, I noticed that some strain L cells in one flask had developed large vacuoles in their cytoplasm. We have grown these cells as a separate substrain in which 15 to 20 percent of the cells are vacuolated. The vacuoles do not interfere with the rate of growth, which is as rapid as of any other substrain of L cells in our laboratory. No abnormalities whatever are observed in the process of nuclear division. If a single large vacuole is present in the cytoplasm of a dividing cell (figures 1 and 2, plate 73) it will go to one of the daughter cells; if more vacuoles are present, they will segregate at random to both daughter cells. Where a very large vacuole is present, the nucleus, but not the cytoplasm, divides. Whether or not this substrain of strain L cells will continue to grow remains to be seen.

PLATE 73

FIGURES 1 and 2.—Dividing vacuolated strain L cells.

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Dr. Lumsden: Dr. Brues made a reference to trephones in his paper. Also, there has been considerable mention, throughout the discussion, of the action of growth-stimulating substances that may be related to cancer growth. The original work on trephones, begun with Carrel, has, of course, led in recent years to considerable development in the identification of the polypeptides and proteoses concerned with the *inflammatory response*. In regard to the cancer problem, it has been considered that such substances, derived from cancer cells, might be concerned in the stimulation of the vascular changes. But the problem is obviously complicated. It is not just a question of stimulation of blood-vessel formation, for once the blood vessels are formed, they condition the improved physiological conditions and thereby lead to overgrowth. A good instance of this direct physiological effect is the hypertrophy that follows artificial anastomoses between the arterial and venous circulations. The effect of substances derived from necrotic cells and stimulating the hyperplasia of endothelium has been invoked especially in the instance of the "glomeruloid" change of blood capillaries in glioblastomas. I tried to investigate this, experimentally, to some extent by making extracts and homogenates of glioblastomas and applying these to cultures of mesodermal cells derived from biopsies of normal adult brain. Similar experiments were made with homogenates of normal adult human brain, as controls, on the same types of cultures. No differences whatsoever in the cultural response of the normal mesodermal cells were detected between those fed with tumor and those fed with normal brain homogenates.

Dr. Braun's presentation makes me wonder whether I have approached this experiment in the wrong way and whether I should have tested for growth-stimulating effects upon glial tumor cells of a *lower degree of dedifferentiation*, *i.e.*, a lower degree of malignancy. In that case the specificity factors he has discussed would perhaps have been eliminated.

Dr. Algire: It is well known that tumors may become vascularized and kill the host even though immune reactions of the host against the tumor are demonstrable. As Dr. Earle pointed out, both the high and the low lines are antigenic and reactions can be demonstrated against both. Vascular reactions are not a primary factor in the destruction of homologous tissue grafts.

Cancer Studies in Fields Collateral to Tissue Culture [1,2]

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Having been asked to introduce this discussion, I first tried very hard to arrive at a definition that would cover "cancer studies in fields collateral to tissue culture." This was not an easy task and the final result was not very illuminating. I thought that I should probably have to deal with experiments of many different kinds performed on living tumor cells under more or less controlled conditions *in vivo*. This would include a number of rather unrelated investigations, *e.g.*, work with transparent chambers, diffusion chambers, and studies on the growth characteristics of tumor cells in the cheek pouch of the hamster, in the anterior chamber of the eye, or in the ascites form. Having arrived at this list I felt that I ought to talk about something different, the reason being that other people would be present who were more competent to talk about these topics than I, and also because I understood that I was not supposed to review well-established techniques and discuss their special merits, most of them already well known, but rather to present some concepts for discussion, no matter how preliminary or controversial they might be.

As a more suitable alternative I have chosen to discuss the phenomenon that is usually referred to as "tumor progression." The meaning of this term was defined and the field surveyed by Foulds (*1*). He includes under this concept all kinds of stable, irreversible, and heritable changes in one or more characteristics of an established tumor. There were three reasons for choosing this topic for discussion; first, because it represents an evolutionary phenomenon not unlike some of the progressive changes that occur in long-maintained tissue culture strains *in vitro;* second, because some cases of tumor progression represent perhaps the most important challenges to tumor therapy at the present moment; third, because, as I shall discuss later, intelligent exploitation of representative stages during the evolution of a given tumor may provide a profitable new field for tissue-culture workers, in which the combination of suitable *in vivo* and *in vitro* techniques may lead to important developments.

There are many examples of tumor progression and I shall not attempt

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[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8-12, 1956.

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to survey them all in a limited time. The induction of mouse pituitary tumors by thyroidectomy, as studied by Furth and his collaborators (*2*), may be quoted as an illustration. Surgical or radiation thyroidectomy results in an increased production of thyrotrophic hormone and the enlargement of the pituitary gland. Transplantation of the enlarged pituitary to a normal mouse of the same isogenic strain does not result in the progressive growth of the transplanted tissue. However, transplantation to a thyroidectomized host leads to expansive tumorous growth. In this "conditioned" host the pituitary behaves like a malignant tumor—it grows to a large size and finally kills the animal. It still differs from most malignant neoplasms because its growth can be checked at any moment by the administration of thyroid hormone. In the terminology of Furth, it is a conditioned tumor. After being carried by serial transplantation in thyroidectomized hosts, such tumors usually change after a variable period of time. As a rule, the change is manifested by the fact that one of the normal, unconditioned animals grows the tumor. When this variant tumor is subsequently tested in normal mice, it usually grows in all of them and can no longer be inhibited by thyroid hormone. At the same time its ability to produce thyrotrophic hormone decreases and its structure changes from a more differentiated to a more anaplastic type. In the terminology of Furth the conditioned tumor has become autonomous. This is a typical case of tumor progression.

Many other more or less analogous changes have been described. Some of them may occur in the primary host of the tumor, others occur only under prolonged transplantation, and some of them may even be regarded as artifacts created by serial transplantation. But they all agree in one respect: They lead in a direction that is usually called increased malignancy; *i.e.*, an increased chance for the tumor cells to grow progressively under the given environmental conditions and a decreased life expectancy for the host. Slowly growing tumors usually increase their rate of growth in the course of serial transplantation; highly differentiated tumors lose most if not all visible signs of differentiation; tumors that are only transplantable within their own inbred strain become transplantable to foreign strains; tumors that can be influenced by certain hormones lose their responsiveness; tumors sensitive to certain drugs develop resistance in the course of prolonged treatment. It would be important to know the mechanism or mechanisms of all these changes. Very few cases have been analyzed as yet in terms of population dynamics. The ones that have been studied represent exceptionally favorable cases from the experimental point of view, although they are perhaps not the most important ones from the practical point of view. They could be analyzed because they occur under controlled experimental conditions and in a reproducible way and because it was possible to develop sublines of the *same* tumor that differ with regard to the particular characteristic under study. I should like to review some of these studies very briefly.

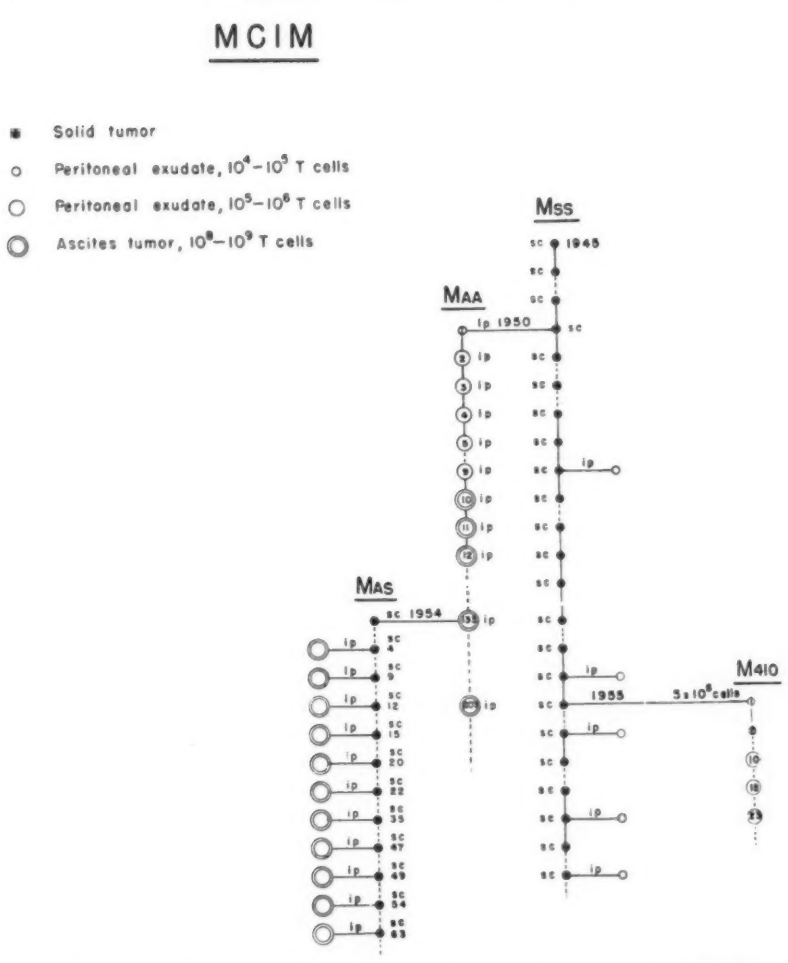
We have been interested in the convertibility of solid tumors into ascites form and have compared 42 different mouse neoplasms with regard to this

characteristic (*3*). Convertibility is defined as the ability of intraperitoneally implanted tumor cells to proliferate as suspensions of free cells and/or clumps of cells in the peritoneal fluid and to reach there a high absolute level (of the order of 10^8 to 10^9) and relative concentration (70 to 90% of all cell types). Convertibility is a variable characteristic, differing for different populations of tumor cells. Once acquired, the characteristic is stable, irreversible, and heritable. The development of convertibility in a previously inconvertible population represents a typical case of tumor progression.

In our material 30 of the 42 neoplasms tested were found to be inconvertible. This group included all more differentiated and slowly growing tumors that were represented. The 12 tumors that proved to be convertible were all rapidly growing and anaplastic. However, there were some rapidly growing and anaplastic tumors in the inconvertible group also. This would indicate that rapid growth rate and/or a low degree of differentiation seem to be necessary, but in themselves are not sufficient prerequisites for the development of convertibility. Some other cellular characteristic is also required.

Among the 12 convertible tumors, 6 could be converted immediately, *i.e.*, a suspension of cells from the solid tumor gave rise to typical ascites tumors immediately after the first intraperitoneal inoculation. Six other neoplasms were not convertible in the beginning, but they could be "trained" to grow in the fluid by prolonged selective transfer. This gradual conversion was highly reproducible for a given tumor and has therefore been chosen for analysis.

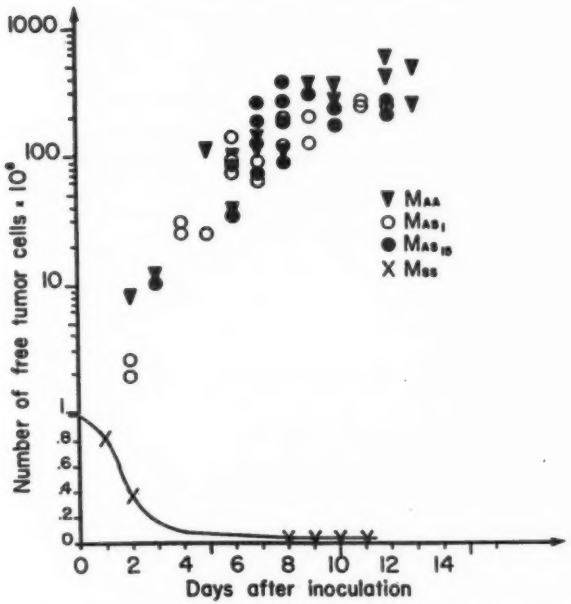
Text-figure 1 shows the history of the gradually convertible MC1M sarcoma. This tumor was induced in C3H mice by methylcholanthrene and its original line, called M_{SS}, has been maintained by subcutaneous solid transfers since 1945. After intraperitoneal inoculation, M_{SS} cells grow in various solid tissues of the abdominal cavity in the form of compact solid masses. Growth is followed by the production of a hemorrhagic exudate that is usually very poor in tumor cells (total number 10^4 to 10^6). If this exudate is carried serially by subsequent intraperitoneal inoculations, the tumor becomes converted to the ascites form after about 10 passages, *i.e.*, its cells attain a total number of 10^8 to 10^9 in the fluid (M_{AA} line). If the converted cell suspensions are reinoculated subcutaneously they grow out as solid tumors again. We have carried such re-established solid lines (M_{AS} lines) serially in the subcutaneous space through many generations. The M_{AS} tumors are similar to the original M_{SS} in many respects. They resemble each other with regard to general histology, cytology, nucleic acid content, nuclear size, chromosome number, and histocompatibility requirements. But they differ sharply in one respect: M_{AS} always gives rise to ascites tumors immediately after intraperitoneal inoculation, even after being carried in the solid form for 67 passages, while M_{SS} regularly fails to do so. This shows that the capacity to grow in the peritoneal fluid, once it has been developed in a population, is stable and irreversible, even if the population is carried in the solid form



TEXT-FIGURE 1.—Transplantation history of the MC1M sarcoma sublines. For M_{AA}, M_{AS}, and M_{410}, the figures denote the number of serial passages.

for prolonged periods of time. Text-figure 2 illustrates the growth curves of the M_{AS} and M_{SS} lines after their inoculation, in identical numbers, into the peritoneal cavity in the form of artificial suspensions.

Figure 1, plate 74, is a phase-contrast image of the exudate that accompanies the growth of the M_{SS} cell type in the peritoneal cavity. The field contains erythrocytes and a few nontumorous exudate cells. No tumor cells are present in this field but if one examines many fields a few cells may be found. Figure 2, plate 74, shows the M_{AA} subline of the same tumor after intraperitoneal inoculation, an almost pure culture of free tumor cells. Table 1 shows the corresponding histological findings in the solid intra-abdominal tissues (4). In this instance we are dealing with an inverted



TEXT-FIGURE 2.—Total number of free tumor cells in the peritoneal fluid as a function of time after the inoculation of 1 million eosin-unstained cells, derived from the original (M_{SS}) and the ascites-adapted (M_{AA} and M_{AS}) sublines of the MC1M sarcoma. Each point represents one mouse. Arithmetic scale 0 to 10^6, logarithmic scale 10^5 to 10^9.

counterpart of the picture in the peritoneal fluid. With the M_{SS} line there is a very intense progressive growth of solid tumor in the cavity, while the M_{AA} and M_{AS} lines show only very small foci of tumorous infiltration, with the largest diameter usually less than 0.25 mm. These appear as early as the implants of the M_{SS} line, but they do not increase in size to the same extent and show only very limited growth. This appears to indicate that the M_{AA} and M_{AS} cells not only grow better in suspension than M_{SS} but they actually prefer to grow in suspension when they are given the choice.

The solid M_{SS} and M_{AS} lines also differ in their ability to metastasize. This can be demonstrated in two ways—by a biological test and histologically. Suspensions of M_{SS} and M_{AS} cells were inoculated intramuscularly in equal numbers. Both cell types give rise to solid tumors that grow at approximately equal rates. To determine the time when metastatic tumor cells appeared in the lungs, the tumor-bearing animals were killed at various times after inoculation and their lungs were transferred subcutaneously to new C3H mice. As shown in table 2, cells of the M_{AS} type gave positive results earlier and more regularly than M_{SS} cells. Recent histological studies (*4*) have confirmed these findings and they have also shown that the two sublines differ even with regard to the morpho-

TABLE 1.—*Histological findings on intra-abdominal tumorous infiltration after intraperitoneal inoculation of 1 million tumor cells from the various MC1M sublines**

Days after inoculation	M_{SS}	M_{AA}	$M_{AS_{20-56}}$
1	−		
2	+		
3		−	+
4	++	±	+
5	++	+	+
6	++	+	+
7	+++	+	+
8	+++	+	+
9	++++	+	+
10	++++	+	+
11	++++	+	+
12	++++	+	+
13	++++	+	

*± Groups of inflammatory cells, presence of tumor cells uncertain.
\+ Small foci of tumorous infiltration, with largest diameter less than 0.25 mm.
++ Implants of tumor tissue between 0.25 and 1 mm. in diameter or very numerous smaller implants.
+++ Implants between 1 and 5 mm. in diameter.
++++ Implants with a diameter of 5 mm. or more.

TABLE 2.—*Tumor transmission with lung tissue from mice bearing intramuscular* M_{SS} *and* M_{AS} *tumors from an inoculum of 1 million cells*

Tumor line	Age of solid intramuscular tumor (*days*)	No. mice inoculated with lung tissue	No. mice developing tumors	Ratio between columns 4/3
1	2	3	4	5
M_{SS}	4	5	0	0. 0
M_{SS}	5	6	0	0. 0
M_{SS}	7	18	3	0. 17
M_{SS}	9	6	0	0. 0
M_{SS}	12	8	4	0. 5
M_{SS}	13	6	2	0. 31
M_{SS}	18	6	6	1. 0
M_{AS_1}	3	10	3	0. 3
M_{AS_1}	6	8	8	1. 0
M_{AS_1}	8	15	15	1. 0
M_{AS_1}	9	8	8	1. 0
M_{AS_1}	13	6	6	1. 0
M_{AS_1}	14	6	6	1. 0

logical picture of the established metastases. M_{SS} cells occasionally produce compact intravascular emboli of tumor tissue in the small vessels of the lung at a late stage during the course of their growth. Subsequently these foci grow expansively. In contrast, M_{AS} disseminates

extensively in the lung tissue, mostly in the form of free, dissociated tumor cells.

It would be of interest to learn something about the chemical or physical correlates of the differences in biological behavior of M_{SS} and M_{AS} cells. We have performed a series of experiments in trying to approach this question, but most of them gave negative results. One that has given positive results of interest was carried out in collaboration with Dr. E. J. Ambrose, at the Chester Beatty Research Institute in London. Dr. Ambrose and his collaborators have constructed an apparatus to measure the mobility of individual free cells in an electrical field (*5*). From such measurements they have calculated the electrical surface charge of their cells under the particular environmental conditions. They found that red blood cells have a very high negative surface charge, while kidney tissue cells have a much lower charge and kidney tumor cells are intermediary. They related these results to differences in cellular adhesiveness; a low surface charge means a high degree of adhesiveness and *vice versa*. M_{AS} and M_{SS} cells are known to differ in adhesiveness. Besides histological evidence, experimental evidence also shows this (*6*). One gm. of fresh tumor tissue contains approximately the same number of tumor cells in both tumors, as determined by deoxyribonucleic acid (DNA) estimation. If artificial cell suspensions are prepared by pressing the tumors through a series of stainless-steel screens of decreasing pore size, the yield of free cells with M_{AS} is more than twice that with M_{SS}. This is the result of the selective elimination of adhesive cell clumps from M_{SS}. In contrast, M_{AS} breaks up easily into individual cells.

Ambrose now has measured the electrophoretic mobility of both cell types under identical environmental conditions and has found a regular and reproducible difference. M_{AS} has a high surface charge similar to and even exceeding that of red blood cells. M_{SS} has a consistently lower surface charge like that of kidney tumor cells measured previously by Ambrose.

Differences in surface charge and adhesiveness between the two tumor lines are suggestive and of interest but it is doubtful whether they explain all biological differences. Probably they show only one part of the picture. There is evidence to indicate that differences in adhesiveness are not always correlated with convertibility to the ascites form. There are some perfectly good ascites tumors that grow in the peritoneal fluid in the form of very adhesive cell clumps (*7*, *8*). And there are some solid tumors that lend themselves well for the preparation of good artificial free-cell suspensions but, nevertheless, do not, under any circumstances, grow in the ascites form. Yoshida has compared 46 induced rat hepatomas with regard to convertibility (*8*, personal communication). He found that 30 were inconvertible, while the remaining 16 tumors, histologically quite indistinguishable from the former group, could be converted to the ascites form. The convertible group was by no means homogeneous. In the ascites form, there was a conspicuous variation between different tumors, particularly with regard to the ratio of free cells and

cell clumps. Each tumor had its constant and characteristic pattern. Some tumors consisted exclusively of large cell clumps. Others were composed of dissociated free cells. Still others were intermediate between these two extremes. This emphasizes again that reduced adhesiveness cannot be the only decisive criterion that determines convertibility. Some other characteristic(s) are also needed. There are some observations to indicate that the nutritional characteristics of the cells may be of importance. If we measure the growth curves of a series of well-established ascites tumors we usually find that some of them grow continuously until the death of the animal, while others reach an asymptote when a certain maximum population size has been attained. This asymptote differs from tumor to tumor (*9*). On the other hand, Hauschka and also our group have developed diploid and tetraploid sublines from the same original ascites tumor and compared their growth curves. We found that both the diploid and the tetraploid line reached the same final maximum population in terms of cell mass; *i.e.*, there were about twice as many cells in the maximum population of the diploid line as compared with the tetraploid. The differences between *different* tumors under conditions of full immunogenetic compatibility, as contrasted with the close similarity between different sublines of the *same* tumor, suggest the existence of variations in the ability of different populations of tumor cells to survive and grow under the extremely competitive conditions that exist in a well-developed ascites tumor where no nutritional gradients are available and where 10^8 to 10^9 cells have to compete with each other for a limited supply of nutrients. It is, of course, equally possible that different types of tumor cells differ in their ability to resist the accumulation of toxic breakdown products.

In another series of experiments we have been trying to approach the question whether the gradual conversion of a solid tumor to the ascites form can be regarded as an adaptation induced by the environment of the peritoneal fluid or whether we are dealing with the selection of variant cell types. Most of this work has been published (*3*), and it may be sufficient to say that the conclusion has been reached that gradual conversion is primarily a process of selection where pre-existing variant cells are being concentrated. The variants are present already in the original solid tumor but their frequency is very low; in the M_{88} line a tentative estimate gave 4×10^{-7} as the most probable figure. The main evidence for a selection mechanism rests on the fact that by modifying the size of the first intraperitoneal inoculum in the beginning of the conversion experiment it is possible to produce convertible or inconvertible lines of the same original tumor at will. By using a very small inoculum one can change a convertible line into an inconvertible one. Together with other confirmatory evidence (*3*), this suggests the pre-existence of the variants.

Similar selection mechanisms have been shown to be responsible for the development of certain types of drug resistance in mouse leukemias (*10*) and for changes in the chromosome number of some transplanted tumors (*11*). At the present stage it would, nevertheless, be quite unsafe to

generalize and to assume that all cases of tumor progression are based on spontaneous cellular variability followed by selection. In fact, there is one system where a permanent change can be shown to have been induced, in the majority of the cell population, by a specific environment. The system is concerned with changes in the homotransplantability of mouse tumors to genetically foreign animals of the same species. It would appear that such transplantation specificity may change in a number of distinctly different ways. The studies of Hauschka and Levan have indicated that genetically specific tumors may become less specific as a result of the selection of variants with higher chromosome numbers (*12*). We have been studying a different tumor-host system (*13*) in which compatibility between tumor cells and certain host genotypes depends solely upon the allelic substitution at a single gene locus in the tumor cells, the *H-2* (histocompatibility-2) locus. The system is based on the isogenic resistant sublines of mice developed by Snell (*14*). Corresponding groups of such sublines are theoretically identical with regard to their entire genetic constitution but they differ at a single gene locus, *H-2*. This locus may bear a series of possible allelic substitutions, and there is considerable variation between different inbred lines of mice with regard to their *H-2* allele. The *H-2* locus is responsible for the specific type of a group of isoantigens localized in tissue cells and also in red blood cells. Transplantation of normal or tumor tissues between isogenic resistant mice is usually not tolerated, since the host can destroy the graft by an immunological reaction provoked by the antigenic end products of the foreign *H-2* allele in the transplanted cells.

Our experimental system was based on the following considerations: One of the isogenic resistant lines, genetically $H\text{-}2^aH\text{-}2^a$, was crossed with another, of the composition $H\text{-}2^bH\text{-}2^b$. Since the 2 lines were isogenic with the exception of the *H-2* locus, the resulting F_1 hybrid was homozygous for all loci with the exception of *H-2*, which presented the heterozygous condition $H\text{-}2^aH\text{-}2^b$. As the next step we induced tumors in such F_1 mice with methylcholanthrene. Most of these tumors behaved in accordance with the genetic theory of tumor transplantation, *i.e.*, they were transplantable to F_1 hybrids of the same genetic constitution only, but not to either of the parental strains. This is due to the fact that each parental strain is capable of reacting against the isoantigenic products of the foreign *H-2* allele derived from the other parental strain and present in the heterozygous tumor cells. However, if one tumor cell were to mutate from, *e.g.*, $H\text{-}2^aH\text{-}2^b$ to $H\text{-}2^bH\text{-}2^b$, it would become compatible with the $H\text{-}2^bH\text{-}2^b$ parental strain but not with the other parental strain. On testing in the appropriate strain, the mutated cell might be expected to possess an absolute selective advantage that would make it detectable. I do not have time to go into the details of these experiments (*15*) but it may be sufficient to mention that such specific mutations do actually occur in tumor cells and can be detected. Thus the transplantability of mouse tumor cells can apparently change as a result of at least two different mechanisms: by a chromosomal mechanism that is not particularly speci-

fic and by highly specific gene mutations. These are examples rather than alternatives, since a variety of intermediates may occur.

There is still a third and basically different possibility, however, which has also been demonstrated. The observations were made on a system first described by Barrett and Deringer (*16*), who showed that a mouse mammary carcinoma will regularly change its histocompatibility requirements upon passage through an F_1 mouse that has been produced by outcrossing the strain of origin to an unrelated strain. We have analyzed this phenomenon by a similar approach used for studies on the ascites transformation. In contrast to the ascites transformation, however, the results gave a clear indication that in this case we are dealing with a real adaptation, induced in the majority of the tumor cells by some factor present in the host environment of the F_1 mouse (*17*). The evidence is based mainly on experiments in which the tumor-cell population was broken up into very small units before passage through the F_1 mice, and the resulting tumors were tested in order to find out whether the change would be induced regularly in all units. Control experiments were done by mixing the changed and the unchanged cell types in different known proportions in order to see whether or not the behavior of such mixtures is compatible with an interpretation of the results in terms of the selection hypothesis. In the present case they indicated that the change was induced *de novo* during exposure to the environment of the F_1 mouse.

After having discussed cases of mutation, selection, and environmentally induced adaptation, I still do not think that we have exhausted all the theoretical possibilities that may be held responsible for certain cases of tumor progression. As a suggested additional model, I would like to mention our experience with a spontaneous mammary carcinoma of strain A mice, called S3A. Tumors of this type are generally believed to arise as a result of the synergistic action of three separate influences: estrogenic stimulation, the milk factor, and the general genetic background. The established tumors are sometimes dependent on estrogenic stimulation and sometimes not (*18*). S3A seemed to belong to the latter category since it was found to grow equally well in both males and females of the original strain during the earliest part of its history. Our usual inoculum dose was 10^6 cells in these tests. A quite different result was found, however, when only 100 cells were inoculated from the same pool; this small inoculum grew progressively in 89 percent of the females but only in 12 percent of the males. Castration decreased the susceptibility of the females to 100 cells, and castrated males behaved similarly. Thus the paradoxical result is that 100 cells of a certain population of tumor cells are estrogen-dependent, while 1 million cells are not. How can this be explained? We can only speculate at the present moment; it appears possible that these tumor cells depend on hormonal stimulation in the beginning only, before the population has reached a certain critical size. After this has happened, however, growth continues, even in the absence of such stimulation, presumably because stimulating or conditioning factors are released by the tumor cells themselves. If present in

sufficiently high concentration, these factors would then compensate for the previously needed action of the estrogenic hormones. If this interpretation is correct, it would indicate that progression may sometimes occur automatically after the cell population has increased to a certain size and even in the absence of mutation and selection or an adaptive change. While it cannot be considered that this mechanism has been unequivocally demonstrated to exist at present, there are some additional findings that fit into the picture. Thus the existence of conditioning or stimulating factors of the type postulated has been demonstrated experimentally (*19*) by mixing dead and living cells in appropriate proportions.

It would seem that early in their developmental history, mammary carcinomas may require such conditioning factors released from other homologous cells and it is perhaps for this reason that they do not grow in the ascites form (*20*). At a later stage of their evolution they often become convertible to the ascites form, provided that large inocula are used. After being carried in the ascites form for some time, even this requirement often disappears, suddenly or gradually, and the cells grow as dissociated cell suspensions, even if only a very small number is inoculated. This is another example of a graded series of progressional steps during the evolutional history of a malignant tumor.

I should like to conclude by attempting to make some points bearing on problems of tissue culture. Whether they will be useful or not remains to be seen.

It would appear that the cellular characteristic called "malignancy" is a very complex phenomenon. I would be surprised if there were two people present who could agree on the same definition for malignancy. Some would probably give preference to invasiveness, others would stress autonomy, or independence from certain controlling factors, or metastasizability. I am not sure that this difference in opinion is necessarily due to our ignorance only. Perhaps it is due to the nature of malignancy. Comparisons between homologous normal and tumor tissues often reveal interesting differences but usually these differences cannot be generalized and fail to apply to other homologous pairs. Perhaps we should not look for a unified concept of malignancy but rather we should study the various partial characteristics that, together, constitute malignancy as a whole. Dr. Gey mentioned the need of specific pairs for comparison for the study of malignancy in tissue culture. For such comparisons we should perhaps not look for *the* malignant cell and its so-called homologous normal counterpart. We have seen many examples in this meeting indicating that both normal and malignant cell strains undergo a separate evolution of their own in tissue culture. This evolution may proceed in divergent directions, and how can we ever be sure that we have the corresponding normal and malignant cell in our hands? How can we be sure that we have a normal or a malignant cell at all? Failure to grow after inoculation into an animal does not necessarily mean lack of malignancy, since growth may be prevented by immunogenetic incompatibility. This is beautifully

demonstrated by the irradiation experiments of Dr. Sanford and Dr. Earle. On the other hand, are we justified in calling a cell strain malignant if it grows after inoculation into a cortisonized or X-ray treated hamster, an entirely heterologous animal that has been neutralized with regard to all its mechanisms of defense and may not possess the homologous growth-controlling factors that would be required to regulate the division of the particular cells that have been inoculated, cells previously stimulated and selected for maximum growth *in vitro*? As an alternative approach, I would suggest tentatively that closer contact be established between studies of cellular behavior *in vitro* and *in vivo*. The stepwise nature of tumor progression provides suitable pairs for specific comparison. You might study one and the same tumor, *e.g.*, in its hormone dependent and independent forms in tissue culture. It is possible to compare 2 sublines of 1 sarcoma, convertible and inconvertible to the ascites form, respectively, and differing in adhesiveness and metastasizability. One may choose sublines of the same leukemia that are sensitive and resistant to a given drug. A series of stages is available during the evolution of mammary carcinomas; prototypes could be chosen that are dependent on estrogen, independent of estrogen but dependent on conditioning factors released from other cells, or independent of conditioning factors. These are only a few examples and many others could be cited.

How can we keep our representative tumor types unchanged? At this point I should like to come back to Dr. Weller's suggestion about freezing. We have had experience with a frozen-tumor bank (*21*) which has been in operation for 2 years. I am afraid I don't have time to show you the results in detail, but it is sufficient to mention that a large variety of mouse tumors can be maintained unchanged at —79° C. for prolonged periods of time.

Finally, I should like to suggest that it might be a more fruitful approach to try to adapt tissue-culture media to the requirements of the different cell types than to adapt all kinds of cell populations to a few existing tissue-culture media. If different cells differ with regard to the media they require, it is the more interesting. Perhaps we should endeavor to develop all the media that various cells require, which would probably mean that most of them would be nonsynthetic. This could be done only if some technique could be used for the mass isolation and identification of single-cell progeny, perhaps of the type described by Puck and his collaborators. Such techniques, if used hand-in-hand with *in vivo* studies on the biological characteristics of the different cell clones, may narrow down the variability between cell strains to the particular characteristic under study. If suitably chosen, these characteristics may be relevant to the complex problem of malignancy.

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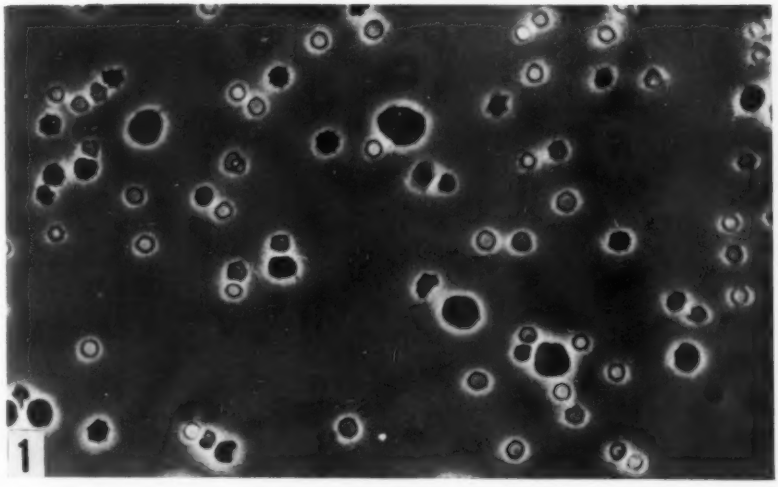
PLATE 74

FIGURE 1.—Peritoneal exudate from a C3H mouse 10 days after the inoculation of 10^6 M_{SS} cells. The field contains erythrocytes and a few nontumorous nucleated cells. Phase contrast. × 900

FIGURE 2.—Ascites from a C3H mouse 10 days after the inoculation of 10^6 M_{AA} cells. Nearly pure culture of free tumor cells. Phase contrast. × 900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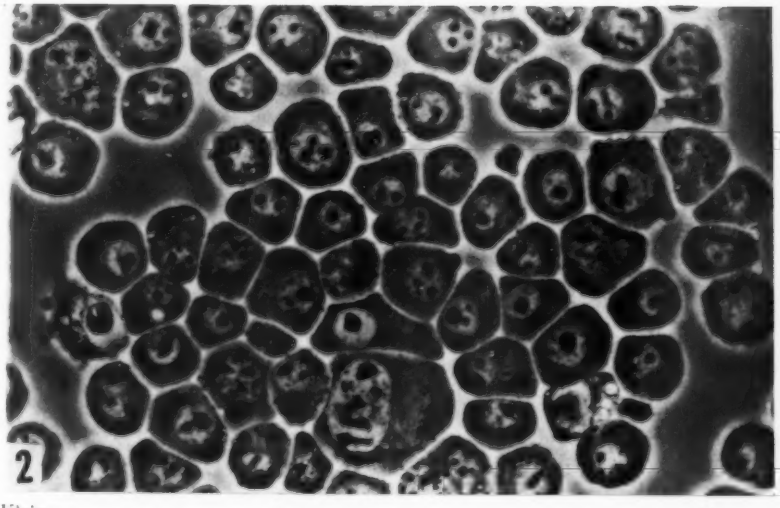
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DISCUSSION

Dr. Lumsden: From Percivall Pott to the Pullmans, *i.e.*, from soot cancer to carcinogenicity in terms of electron density at the phenanthrene double bond, the chemical induction of cancer is still the most impressive monument in cancer research. Viruses can induce cancer and, as Kidd showed, can die out leaving the cancer. The evil that these exogenous carcinogens do lives after them. In view of this and of the dual mutagenic and carcinogenic potentialities of irradiations, it was obvious that somatic mutation seemed the answer to the cancer problem.

There are, as I see it, possible objections to mutation as a general hypothesis for human cancers, whatever its role in the special varieties of experimental cancer we have just heard about. First, with human cancers, there is their great frequency and later appearance compared with other mutations and mutation-linked diseases like albinism, hemophilia, porphyria, cystinuria, and Lever's optic atrophy. Second, the cancer change is not necessarily only in the progeny of the cells exposed to the carcinogen. Thus, adult skeletal-muscle cells, adult nerve cells, and probably even astrocytes, have all lost their power to multiply in numbers in response to other pathological stimuli, but tumors can be induced from them with methylcholanthrene. We must, therefore, assume cancerous change in some of the actual cells exposed and not just in the derived progeny. Third, unlike known mutations produced experimentally, the changes within the cancerized cells are not abrupt; and we owe to Berenblum and to others in recent years the patient re-evaluation of the essential role of the *promoting* as well as of the *initiating* factor. Such promoting factors are not necessary in mutations, at least as far as we know. Fourth, there is evidence sometimes for reversibility of the cancerous change, as in the behavior of some prostatic primaries and secondaries following castration. Dr. Klein cited other examples. Fifth, many human cancers are evidently multicentric in origin, as in the familiar examples from lip, tongue, ducts of the breast, rectal polyposis, and some pigmented nevi. Experimentally, mice treated with urethan may develop hundreds of tumors in different parts of the lungs, all apparently primaries.

Undoubtedly, cancer cells have genetic factors, as in the instances given by Dr. Klein and some of the plant tumors discussed earlier today. Alternatively, this might be stated in another form, *viz.*, that *cancer cells are not necessarily liberated of all their genetic factors.* Some of their behavior in this respect undoubtedly accounts for the variation in transplantability in animal work, including some results of experiments with the anterior chamber of the eye.

It is difficult to see how the mutation hypothesis could ever be proved experimentally, since we cannot wed a cancer cell to a normal cousin and observe the progeny. But I think it might be possible to design an experiment to disprove it by taking 100 isolated fibroblasts, each in a capillary tube, exposing them to an appropriate dose of carcinogen, cloning them, and finding out by animal inoculation what percentage of clones eventually proves malignant. A significantly high incidence of cancer, as against the controls, would, to my mind, exclude mutation in its strict sense.

An even more fruitful attack by tissue-culture methods might be upon the *physical properties* of the cancer cell rather than upon its "cause." Many regard "cancerism" as a single property, a new somatic gene, or a "rogue" enzyme that is added to the cell and confers upon it its attributes of uncontrolled growth, invasiveness, and metastasizability all in one. I think these last attributes are separate and separable properties of the malignant cell. Thus, what is often forgotten by cytologists is that the histopathologist's evaluation of these 3 components of malignancy is not only a composite one but one that has to be related specifically to each organ or epithelium concerned. An irregular cellular architecture of the duct epithelium, which is harmless

in the prostate, spells malignancy in the breast. We may contrast, too, the vital difference of the hyperplasia in a wart with the same degree of hyperplasia in a pigmented nevus. Again, invasion of vein walls in any tumor except a hydatidiform mole means malignancy with the probable occurrence of metastasis; but its correct evaluation in a uterine scraping may prevent unnecessary mutilation.

Metastasis itself is a compound of many factors. Simple dissemination of cells by the blood stream does not necessarily mean much. Examples of normal cells disseminating in this way can be given, *e.g.*, bone marrow or liver cells following trauma. In the case of tumor-cell embolization via the blood stream, purely hydrostatic pressures are probably an important determinant (*1*). Certainly, tissue pressures are consistently raised and continue to rise with time, being increased by manipulation in the Brown-Pearce carcinoma of the rabbit testis (text-fig. 1, p. 814), as compared with the surrounding tissue or with those of the normal contralateral organ, as our results showed (*2*). While it is true that the tumor cells may have greater proteolytic action upon ground tissue, invasiveness, too, may be the result of physical phenomena. With model systems involving the use of gelatin beads or "cells" of various densities and simulated vessels, also of gelatin, Young has been able to show how, simply by increasing the interstitial pressure, differential movements of "cells" with higher densities occurred progressively between "normal" cells with lower densities (*3*). Even "penetration" through the walls of the gelatin "vessels" occurred. Thus, both lymphatic permeation and invasiveness with embolization were simulated in a simple physical system.

Malignancy is therefore in my view not a single genetic property but a composite result. What is its chief component? My guess is that it is the physical change in the cytoplasm. I think it is here that one of the greatest potentialities of tissue culture lies because of the possibilities, with this method, of seeing the whole cell and of testing instrumentally the physical characteristics of its components.

How closely do the cytoplasmic characteristics of the tumor cells, in culture, resemble those of the normal; and what changes in these characteristics occur with increasing malignancy? These two problems can be most effectively studied, in my experience (*4*), in neuroglial cancer in man, because (*1*) tumors of the astrocytoma-glioblastoma series form such a well-graded, continuous series and (*2*) the neuroglial cell retains, even in neoplasia, such a kenspeckle cytoplasmic organization, even in the higher grades of malignancy (fig. 1, plate 75).

It is, it seems, in the alterations of membrane mobility, in the changes of adhesivity of the cell surfaces, and in the lowered viscosity and increased density of the cytoplasm that some of the chief qualities of malignant cells may reside that are responsible for part of their behavior. In the ability of tumor emboli to seed in metastatic sites, problems of immunological specificity are probably involved also. But some of the malignant changes have, perhaps, an essentially physical basis only. And one may go further and ask whether the physical changes, physically induced—as, for instance, the prolonged growth of cells on glass surfaces—might not be capable of initiating some of the critical biochemical changes such as those of nucleoprotein synthesis that have been discussed at this meeting. Is it not true that a mere mechanical or osmotic stimulus can induce division in a sea urchin's egg?

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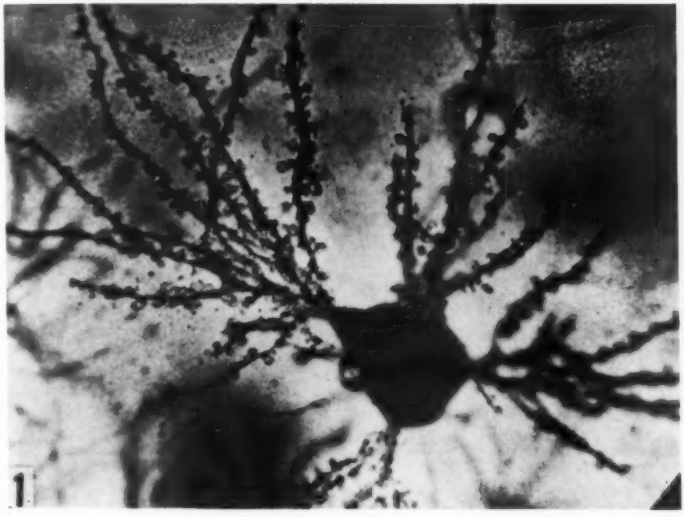
PLATE 75

FIGURE 1.—Neoplastic astrocyte in tissue culture. From fairly highly malignant astrocytoma. This epitomizes the problem of the morphological "dedifferentiation" of cells of this family, *viz.*: the processes are increased and arise from "cones" of perikaryon, are shorter than normal astrocyte processes in culture, and possessed of characteristically rich lateral pseudopod-like extrusions of cytoplasm along the entire length of the processes as well as the tips.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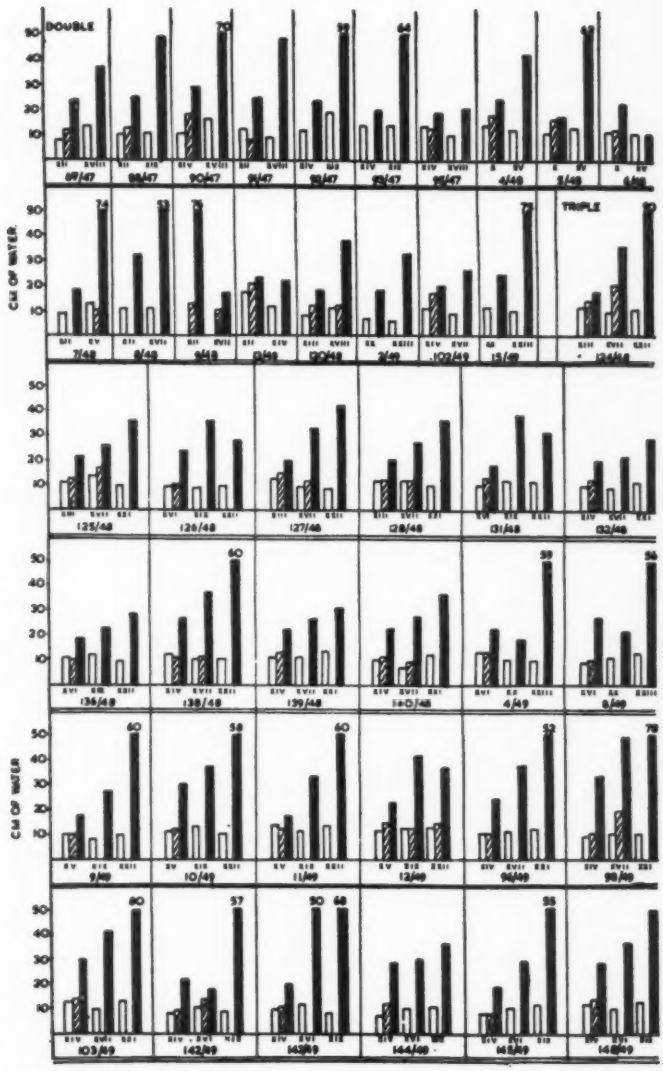
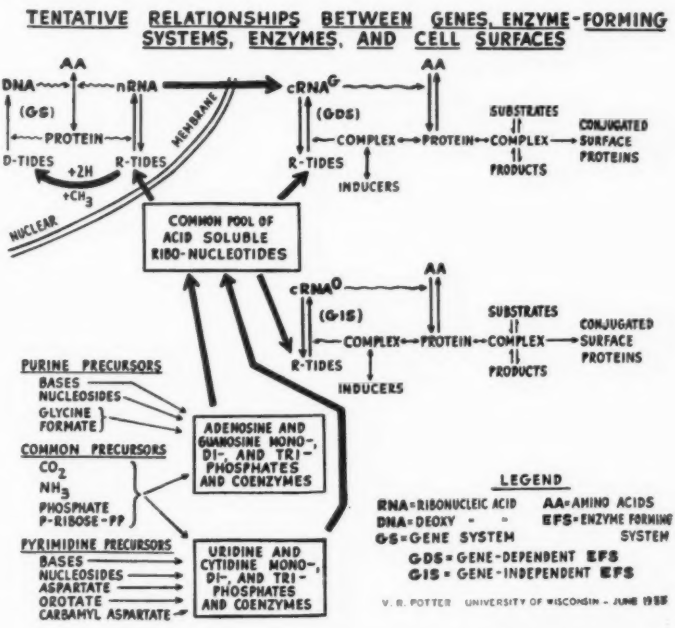


TEXT-FIGURE 1.—Serial estimations, *i.e.*. 18 double and 25 triple, recorded in 43 rabbits: *white*, normal testis; *hatched*, testicular tissue investing tumor; *black*, tumor. Arabic numerals below = rabbit number; roman numerals = day of estimation (after implantation of tumor); arabic numerals above = tumor-tissue pressures exceeding 50 cm. of water. (Reproduced by permission of editor, *J. Path. & Bact.*)

Dr. Potter: Whenever I hear Dr. Klein speak about the cancer problem, I have renewed optimism about the ultimate solution of the problem. I believe, as he does, that there are both nuclear and cytoplasmic factors involved, although I may emphasize the cytoplasmic factors to somewhat greater extent here. I believe that we are ready to consider a collateral area of study involving cell-free systems. This

may be called intracellular physiology. The fact is that we are beginning to have some success in preparing suspensions of nuclei, mitochondria, fragmented cytoplasmic membranes, and suspensions of ribonucleoprotein particles. It is now possible to combine parts of tumor cells with parts of normal cells and to show, for example, that the liver mitochondria can suppress tumor glycolysis (J. Biol. Chem. 224: 1099 and 1115, 1957). It is also possible to carry out, in cell-free systems, multiple reactions that lead to the labeling of ribonucleoprotein with isotopic nucleic acid precursors. We believe that the reactions represent the beginnings of true nucleoprotein biosynthesis in such cell-free systems.

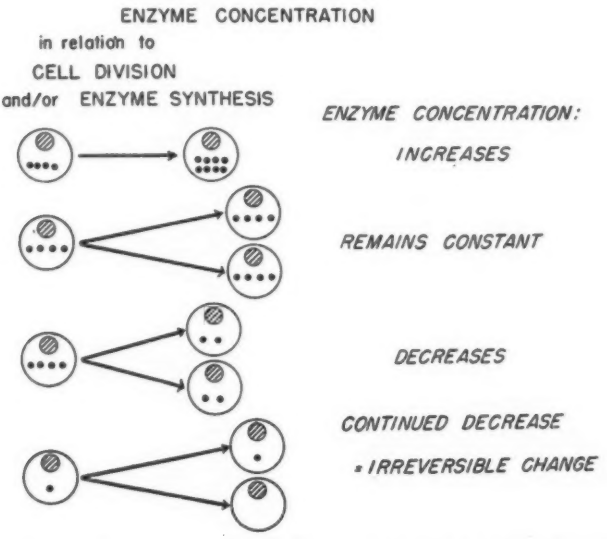
The type of system that we are dealing with is shown in text-figure 1. In this figure I have omitted certain things such as the reactions of the mitochondria and the glycolytic system—in other words, the energy-generating systems. I wish to discuss two features. One is the acid-soluble fraction, in which I would like to emphasize the principle of multiple alternative metabolic pathways. Different enzymes for each substance can lead to many different products. We also have to consider converging pathways for an individual metabolite. The other principal part of the hypothesis of cancer that I wish to present is the ribonucleoproteins, virus-like particles that occur in normal cytoplasm. In other words, we are concerned with the ribonucleoprotein particles as intermediaries between genes and enzymes and with the acid-soluble, small-molecule metabolites on which the enzymes act. A number of these nucleotides were found in our laboratory and reported in 1954. We know now that there are at least 12 nucleotides that are intimately related, as building blocks, to the ribonucleoproteins. These ribonucleoprotein particles occur in what used to be called the microsome fraction, which was the term applied to the class of particles in the homogenate after the nuclei and mitochondria had been removed. It is now known that the microsomes do not occur as such in the cytoplasm; they are formed by the breakup of the reticular network that occurs in the cytoplasm. This network



TEXT-FIGURE 1.—Diagram of nucleoprotein metabolism.

has in it a number of very small particles that are ribonucleoproteins and that might well be called Palade particles. The network in the cytoplasm appears to break up when the cells are broken and then reconstitute itself into smaller, hollow spheres in the homogenate, just as the filamentous mitochondria appear to round up in the homogenate, while the ribonucleoprotein particles continue their previous association with the membranes of the reticulum. It is apparently a basic property of protoplasm that it can survive considerable mechanical damage if its chemical requirements are met. These chemical requirements include a continuous supply of adenosinetriphosphate (ATP).

The importance of these ribonucleoprotein particles is believed to be as follows: 1) They are apparently the basis of enzyme synthesis or the enzyme-forming system referred to by Lwoff, Spiegelman, and other people working in that area. 2) These particles appear to be self-duplicating. Text-figure 2 is from a former study in which we did not refer to the enzyme-forming system but to enzyme synthesis. The particles appear to be self-duplicating at independent rates and at rates that are not identical with cell division. 3) They are particulate and occur in small enough numbers to make it seem likely that each enzyme is represented by relatively few particles. 4) There is no *known* mechanism for regulating their division between the two daughter cells during mitosis, yet unequal partition is assumed and polarizing mechanisms based on enzyme induction can be suggested. 5) When the number of such particles reaches 1 per cell or becomes highly polarized, cell division will result in an irreversible change in one of the daughter cells as in example 4 (lower part of text-fig. 2). 6) If this particle is concerned with deoxyribonucleic acid (DNA) synthesis, the resulting cell will be unable to divide and is started on the way toward differentiation. 7) Differentiation in nondividing cells is believed to result from unequal increase in certain of these particles, as in example 1 (upper part of text-fig. 2). 8) Cancer cells are believed to result from the loss of particles competing with the particles responsible for DNA synthesis, as in mechanism 4 (lower part of text-fig. 2), but a different type of particle than the earlier example. 9) In infections by the ribonucleic acid (RNA) viruses, the virus particles compete with the normal RNA particles.



TEXT-FIGURE 2.—Changes in concentration of cytoplasmic ribonucleoprotein particles relative to cell division. The crosshatched area represents the nucleus.

Since the essence of this mechanism of cell change is the *competition* between particles, let us consider the basis of the competition (text-fig 1). The main point is that all of the particles contain RNA protein. Only the postulated two main classes are shown. There seems to be large numbers of types corresponding to specific enzymes, but all of them are in equilibrium with the acid-soluble pool of 4 ribonucleotides. These 4 nucleotides are uridylic, cytidylic, adenylic, and guanylic acids. The competition is based on the following: 1) a different equilibrium between each of the RNA's and the pool—the RNA^{O} class being irreversible, the RNA^{Q} class being reversible; 2) the presence of inducers or substrates moves the equilibrium in the direction of the RNA protein; 3) viruses are assumed to have equilibria that favor the virus and are disadvantageous to the normal particles; 4) the RNA of the RNA^{Q} class tends to be in balance with the pool and is backed by DNA; 5) RNA of the RNA^{O} class tends to have an equlibrium favoring the RNA and the system is less dependent on DNA. The present status of this type of work is: Cell-free systems are at hand and they are working; it is possible to show the equilibria between these nucleoproteins and the nucleotide pool; it is possible to show in the whole animal that the equilibria are different for different types of RNA; and, finally, there is a report in the literature by F. B. Straub, of Budapest, showing that a penicillinase has been induced in cells in the absence of penicillin with RNA extracted from organisms that have had penicillinase (*see* Biochim. et biophys. acta **20**: 401, 1956).

For the future I believe that there are 2 lines of work that might be mentioned. I am sure we are all tremendously impressed by the magnificent work that Dr. Grobstein is doing. The idea of adding particles prepared from various types of cells to tissue cultures in an attempt to transform one type of cell to another will be tested by other people without urging from me. It will be found in many of these cases that the factors involved are not RNA. Sometimes they will be RNA, sometimes small molecules.

I am impressed by studies of the type reported by Dr. Gaillard and Dr. Moscona. I want to urge that differentiated cells that have lost the power to divide be treated with RNA protein from dividing cells to see whether it will restore the power to divide. It is basic to our entire concept that there are cells which divide and have offspring unlike the parent cells (text-fig. 2). We assume that the differentiated cells which survive in organ culture and which will not divide have lost the cytoplasmic particle necessary for DNA synthesis. It remains to be seen whether the type of culture that is used by Dr. Gaillard and Dr. Moscona can be converted into a system in which these cells will divide by particles obtained from cells that are rich in the missing properties. Finally, I think there is a very real basis for hoping that cell-free systems can be set up in which virus reproduction, RNA synthesis, DNA synthesis, and adaptive enzyme formation can be obtained. If I sound as if I am overselling this proposition, and if this does not come about in 1957 or 1958, we shall not be surprised nor apologetic. I feel that it is necessary to make a strong case for the use of cell-free systems, because this organization contains the people who have much of the training and the background necessary for that type of work in combination with the techniques being developed in my own laboratory.

Dr. Algire: Microscopic studies of normal or neoplastic cells following their implantation into animals frequently are complicated by the difficulty of distinguishing cells of the graft from those of the host, by inflammatory and circulatory reactions, and by resistance phenomena.

We have been able to simplify the complex *in vivo* environment of grafts through the use of porous filters (*1–4*). The filters are sealed to plastic rings and sterilized. The implant or cell suspension is placed between two layers of filter, and the chamber is assembled. The chambers are then implanted under the skin or within the peritoneal cavity of mice and removed later for histologic study. These chambers have been adapted also for microscopic observation of living cells in transparent chambers in mice.

Cells survive and proliferate in these chambers, as a "tissue culture *in vivo*," nourished by diffusion of fluid from the host but having no contact with host tissues. In

studies with Dr. Weaver and Dr. Prehn (*2–4*) it has been found that not only isologous grafts but also homologous grafts survive indefinitely in these chambers. Homografts survive even if the host has been immunized previously.

Heterografts of human cells (HeLa strain) proliferate rapidly, but by 15 days areas of cellular degeneration are found intermingled with healthy cells (*5*). In contrast to homografts, heterografts are quickly destroyed by the circulating antibodies from an immune host. In current experiments, problems of cellular immunity of cancer are being studied with filters having a graded series of pore diameters.

Chambers have been constructed from which single or successive samples of filtrate may be obtained for chemical analysis, antibody titration, or the inoculation of cells in fluid-suspension media (*6*).

Questions are constantly raised as to the reality, in the living animal, of many of the phenomena that have been described from observations of cells in tissue culture.

Diffusion chambers have been adapted to the transparent-chamber method (*1, 6*) in order to observe cellular behavior within living animals by phase-contrast illumination. Time-lapse motion pictures have been made showing migratory activity of various cell types, pinocytosis, mitochondrial movements, and cell division. It has been found that many of the cellular activities that occur in the living animal are very similar to those described by workers in the field of tissue culture (*1, 6*).

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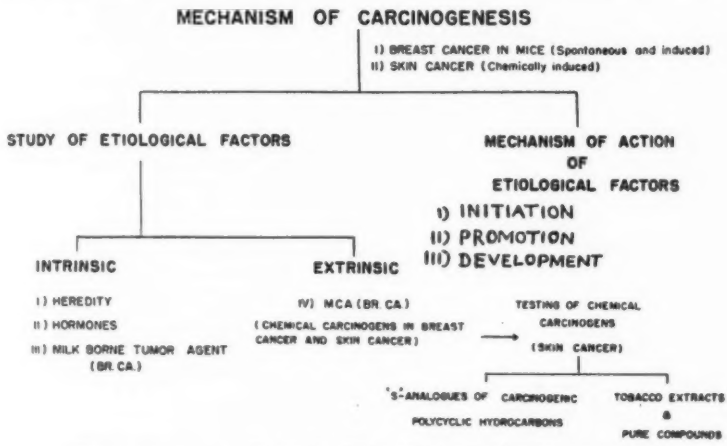
Dr. Ranadive: I would like to present a general outline of my program of biological research in India—both *in vivo* and *in vitro*—in the light of the discussions of these last few days.

In order for you to understand the background of the conditions under which we must work, I should stress that in India, cancer is a problem far down on the list of public-health problems. We are still struggling in our villages with simpler problems like malaria, smallpox, tuberculosis, etc. If it were not for the tenacity and foresight of one man as our world-famous pathologist Dr. Khanolkar, we could not have started cancer research for many years to come. Considering these conditions we certainly are ahead of the schedule. In the last 3 years, we have developed a fairly well equipped laboratory—the Indian Cancer Research Centre in Bombay. The tissue culture department is only 3 years old.

Twelve years ago, when I joined the laboratories of the Tata Memorial Hospital, we became greatly interested in the literature of Stout and Murray and wanted to use tissue culture as an aid in diagnosis and problems in histogenesis. We soon discovered the practical difficulties in our way. Every ingredient in the balanced salt solution and every Maximow slide and coverslip had to be imported from foreign lands. Those were the days when the second World War was in full swing, and we therefore had to

give up the idea of starting this fastidious technique and satisfy ourselves with purely histological work on inbred strains of mice imported from Bar Harbor and Yale.



TEXT-FIGURE 1.—General outline of the work under way *in vivo*.

Text-figure 1 gives the general outline of the biological work planned in our laboratory for study of the mechanism of carcinogenesis. The work has been concentrated mainly on two cancer types—breast cancer in mice (spontaneous and chemically induced) and skin cancer (chemically induced). The project has been divided in two parts: 1) a study of the etiological factors—*a*) intrinsic and *b*) extrinsic—and 2) an investigation of the mechanism of the process of carcinogenesis by study of the interaction of different factors causing initiation, promotion, and development of the disease. It was planned to study the comparative morphology and histology of mammary glands and the endocrine system, in different experiments designed to investigate interrelationships and interactions of various intrinsic and extrinsic factors in the process of carcinogenesis. Careful studies of some 7,000 preparations of mammary glands and endocrines confirmed the role of the milk-borne tumor agent (virus) as an initiator of spontaneous carcinogenesis and the hormones as promoters of the disease on genetically susceptible soil. Our original contribution in these studies was the influence of the milk-borne agent on hormonal metabolism, indicated by changes in the sex cycle and the structure of ovaries and adrenals after foster nursing, in intact as well as in ovariectomized mice of different strains. Comparative study of two lines of the same strain, with and without the milk factor, demonstrated significant differences in ovaries and adrenals. Mice susceptible to cancer that were gonadectomized at birth and resistant-strain castrates foster-nursed on the milk of susceptible animals showed heavy deposition of calcium salts in the stroma of the mammary glands. It was noticed that the adrenocortical hyperplasia in such castrates influenced calcium metabolism.

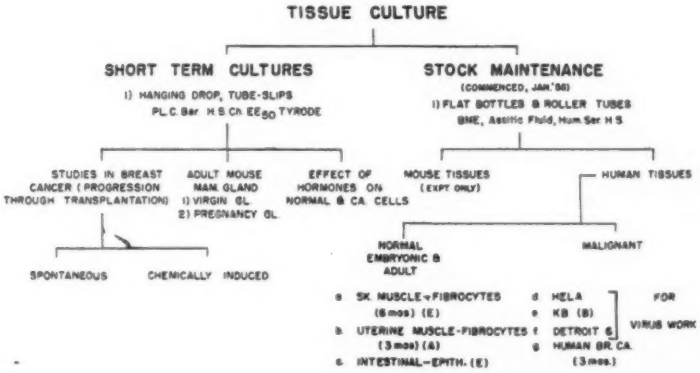
Similar studies on mammary glands and endocrines were continued on 3-methylcholanthrene-treated (20-methylcholanthrene) breeders of different strains of mice and their progeny. We confirmed the role of 3-methylcholanthrene as the initiator. The role of a hormonal factor—especially an adequate level of luteal factor from the ovary in combination with follicular stimulation—was demonstrated to be the promoter of carcinogenesis on genetically susceptible mammary tissue. Progesterone was established as an essential factor in chemical carcinogenesis and the hormone-mimetic action of 3-methylcholanthrene was demonstrated in a new low-tumor strain, L (P).

Induction of skin carcinoma was used as a standard technique to investigate carcinogenicity of test compounds. A series of sulfur isosteres of carcinogenic polycyclic hydrocarbons was investigated to correlate the chemical constitution of a substance with its carcinogenicity. These compounds were tested especially to verify Robinson's hypothesis of an activated phenanthrene bridge. Seven sulfur isosteres of known carcinogens, 7,14-dimethyldibenz[*a*,*i*]anthracene (9,10-dimethyl-1,2,7,8-dibenzanthracene), 7,14-dimethyldibenz[*a*,*h*]anthracene (9,10-dimethyl-1,2,5,6-dibenzanthracene), dibenz[*a*,*h*]anthracene (1,2,5,6-dibenzanthracene), benzo[*c*]phenanthrene (3,4-benzophenanthrene), and chrysene were tested. Four of these did not fit into the activated-phenanthrene-bridge hypothesis. The work on the hormone-mimetic properties of some of these compounds is still in progress.

Another important problem in experimental carcinogenesis, specific to India, is the investigation of the carcinogenicity of sun-cured tobacco leaves. Oral cancer comprises 40 percent of the total cancer cases in our country, and the habit of chewing tobacco is considered to be one of the important contributary factors of this disease. The biochemists at the laboratories therefore have fractionated tobacco by extraction with various solvents, and these fractions are tested by skin painting of the back and buccal mucosa of mice, by subcutaneous injection, by Rous' embryonal method, by inserting pellets in the hamster cheek pouch, etc. No significant carcinogenic effect has been yet noticed at the site of administration.

With this general program in view, of work *in vivo*, our tissue culture section has been organized to use the technique specifically for projects in experimental biology (text-fig. 2).

The program in tissue culture may conveniently be divided in 2 parts: One is the maintenance of experimental short-term cultures and the other is long-term or indefinite maintenance of stock cultures. The chart gives the routine techniques and media used for both short-term cultures and for stock maintenance. The first series of cultures, cultivated by the hanging-drop technique for study of tumor progression through transplantation, was made with spontaneous and 3-methylcholanthrene-induced breast tumors from different strains of mice. A good number of mouse mammary adenocarcinomas, 1 carcinoma simplex, 1 adenoacanthoma, and 1 carcinosarcoma have been studied *in vitro* for over 30 generations. Significant progressive changes in growth rate, growth pattern, and grade of malignancy have been noticed, especially in carcinoma simplex and in a methylcholanthrene-induced carcinoma that was transformed into carcinosarcoma. The carcinoma simplex from a line free of milk factor grew very rapidly with high proteolytic activity and presented characteristic reduced adhesiveness of cells, producing loose epithelial membranes with clear intercellular protoplasmic bridges. This finally developed into a highly



TEXT-FIGURE 2.—General outline of the work under way *in vitro*.

anaplastic carcinoma and appeared to be ideal material for transformation into ascites, according to Dr. Klein's discussion on the subject. Intraperitoneal injections attempted in later generations gave encouraging results. These mouse mammary tumors—15 of those studied so far—are being standardized specifically for use in an experimental chemotherapy program to be undertaken in the near future.

The other tissue grown for experimental purpose is the adult mouse mammary gland grown simultaneously with the mammary tumor for cytological comparison as well as to study the effect of ovarian hormones on the mammary gland. We have not yet used the special modified technique of Dr. Lasfargues for cultivation of mouse mammary gland but our experience so far with the mammary gland at mid-pregnancy is that the epithelial cells grown from this source present more cytological abnormalities than epithelial cells grown from breast tumors. Cytologically, mouse mammary tumor is a particularly regular material; that the mammary gland in pregnancy should present such cytological abnormalities is an interesting finding.

The stock-maintenance work was started only at the beginning of this year and the cell types indicated in text-figure 2, all of human origin, have been maintained. (Mouse-tissue cells are used only for experimental short-term cultures.) As shown in the chart there are 2 tissue types: 1) *normal*, *a*) embryonic, *b*) adult; 2) *malignant*, which is of interest to us in developing our program of work in experimental carcinogenesis and chemotherapy. Human embryonic tissues, both fibroblasts (from human skeletal muscle) and epithelial cells (from intestine), and human adult fibroblasts derived from uterine muscle are available as stock cultures. The commercial human tumor-cell lines, HeLa, KB, and Detroit 6 have been maintained in flat bottles as a duplicate stock for the Virus Research Centre in Poona and are used on "slip cultures" for cytological studies and in chemotherapy. We have both HeLa and KB stock well adapted to horse serum instead of expensive human serum. Cultivation of "human breast cancer" *in vitro* is a problem of specific interest for investigations of various factors *in vivo*. Twenty-five percent of the 35 human breast tumors grown so far in roller tubes have given good yields of pure epithelial membrane, the longest period of maintenance being 3 months.

Normal mouse embryonic and human fetal fibroblasts grown from skeletal muscle have been used invariably in our program for testing carcinogens. Some sulfur isosteres of polycyclic hydrocarbons, as well as pure compounds isolated from tobacco, like γ-sitosterol, which are being tested by *in vivo* experiments, have been screened *in vitro* for carcinogenicity. Although a higher mitotic index and few cytological abnormalities have been observed in some of the cultures treated with the test substances, I am not sure how far one can rely on these purely morphological and cytological differences to distinguish a living malignant cell from a normal, healthy cell—unless, of course, one has beautiful movies like those of Dr. Gey. After listening to the interesting discussion of Dr. Gey and Dr. Earle, I am not sure if the time is yet ripe for a laboratory like mine to use tissue culture as a technique for experimental carcinogenesis. I would rather wait for the older laboratories to get better results to clarify this point of conversion of normal cells into malignant cells *in vitro*. Indeed we are busy standardizing other cytochemical and biophysical methods to give us better insight into the mechanism of conversion of normal cells into malignant cells in experimental carcinogenesis.

The other program in which tissue culture may be used as a screening technique simultaneously with *in vivo* experiments is the chemotherapy program. As in other countries our organic chemists are busy synthesizing enzyme inhibitors. Some antifertility compounds prepared for the family-planning program will be worth testing for growth-inhibiting activity. Besides, there are drugs in ancient Indian medicine—the so-called "Aurvedic system of medicine"—samples of which come pouring in to us for testing. Some of these need thorough investigation. Tissue culture used in combination with animal experiments thus promises to be a valuable tool for different aspects of cancer research.

Dr. Harris: I was particularly interested, Dr. Klein, in what you had to say concerning the preservation of cell lines in the deep freeze. This is a point of great

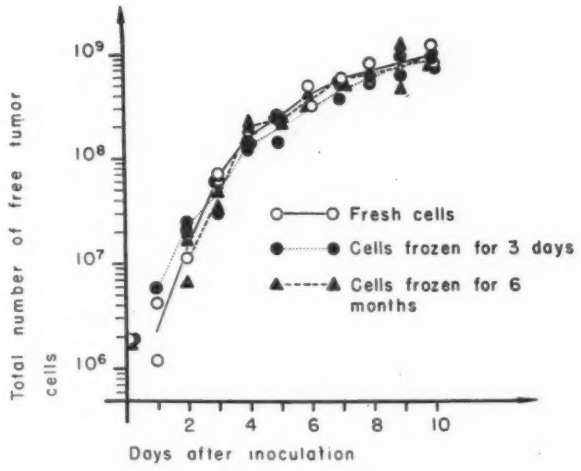
interest in relation to the stability of these lines and whether procedures of this sort might possibly influence it. I think Dr. Morgan might care to comment on this, in relation to some interesting work he has been doing.

Dr. Morgan: We have accidentally obtained observations on the mouse-strain specificity of certain ascites-tumor lines which appear to be rather unusual. We harvested the ascites cells from 2 mouse-strain-specific lines, the Gardner lymphosarcoma 6C3HED and adenocarcinoma TA3. We resuspended these cells in saline containing 20 percent glycerine, froze them rapidly, and stored them at −70° C. When we tested the tumors after this treatment, the mouse-strain specificity had completely disappeared. The formerly specific lines now developed, apparently normally, in any of the mouse strains available in our laboratory. These were rather limited but they did cover a reasonable range of well-known mouse lines and one of very mixed genetic origin. This loss of specificity appeared to occur immediately, there was no obvious selection, and the change appeared to be irreversible. The most curious aspect of all this, and quite an unexpected one, was the fact that the chromosome number of both the specific and the nonspecific sublines appeared to be the same. The cells were diploid before the treatment and seemed to remain so. I know these results are rather contradictory to an extensive literature and I don't propose to attempt to interpret our findings. However, I would like to say that we didn't expect these results and we therefore rechecked and repeated all the experiments 4 separate times. No matter what the interpretations in the future may prove to be, I think the original observations are probably valid.

Dr. Klein: I was very much interested to hear about Dr. Morgan's results. They certainly do not agree with our experience (Klein, G., Révész, L., and Klein, E.: Transplant. Bull. 4 : 3–33, 1957), but there is an important difference in method which may be responsible for this discrepancy. We have had a frozen-tumor bank in operation for about 2 years and have tested about 600 samples from a variety of mouse tumors. So far we have observed no change in any of the following characteristics after freezing and thawing: histocompatibility requirements, transplantation specificity, growth curve, growth rate, dependence on a certain hormone, drug sensitivity or resistance, and convertibility or inconvertibility to the ascites form. In order to find out whether or not the majority of our cell populations survive the procedure, known numbers of ascites-tumor cells were frozen and thawed. The growth curve of the same number of fresh cells was then compared to that of cells stored in the bank for 3 days and to cells that were frozen for 6 months. All cells used for this experiment were derived from the same original pool. Growth curves were measured by determining the total number of free tumor cells in the peritoneal fluid as a function of time after inoculation. To our great surprise we found perfect overlapping (text-fig. 1). I don't think that a difference in the inoculum size of, say, 10 or 20 percent would show up in the growth curve. The method is not sufficiently sensitive to detect small differences but it would certainly show the death of a large proportion of the population. The results can be taken to indicate that the majority of the cells survive the freezing procedure used, at least with this particular tumor. The most important difference between our method and Dr. Morgan's is that we do not add anything to the cells and do not handle them by washing or centrifugation. We do not add either glycerol or any "physiological" solution. The solid or ascites-tumor samples are transferred to small dry tubes as they are removed from the animal and frozen directly at −79° C. If we add balanced salt solutions before freezing the cells usually die. Perhaps we could protect them in glycerol. We haven't tried this because we obtained perfectly good results without adding anything. I am very much interested in Dr. Morgan's results and I wonder whether the changes described by him are, possibly, induced by the conditions of freezing in a saline-glycerol mixture. This would be very interesting, if so.

Dr. Rey: I am most interested in what has been said by Dr. Klein and by Dr. Morgan on low-temperature storage of cells, particularly of tumor cells.

We have some experience of this work in our laboratory in Paris and have found that sometimes there is a decrease in specificity after the exposure to low tempera-



TEXT-FIGURE 1.

tures. It appears that after quick freezing to very low temperatures (dry ice or liquid nitrogen) something like despecification of the specimen occurs. For almost all tissues it is necessary to add some glycerol. Viability is then preserved, but we think that there is a slight alteration of the constituent proteins, either during the freezing and thawing processes or during the storage period, and that is why there occurs a loss in specificity. This may be of great value for the tissue-banking problem.

Dr. Hyatt: Since freezing does seem to be of interest as a method of cell preservation, may I suggest that before we adopt the word "freezing" we adopt qualitative and quantitative expression of freezing velocity. If we are interested in a basic principle involved in cell freezing and storage let us do it with the aid of the thermocouple. Let us specifically design or describe exactly what we mean by freezing. For example, the tissue-bank personnel working with Dr. Earle and Dr. Evans have been able to subject the pure epithelial strain 2414 to a freezing velocity, which can be accomplished with either liquid nitrogen, or dry ice and alcohol slush and storage at −20° C. in the Amana refrigerator for 1 hour, with survival on subculturing. There were no observed differences in morphology. I am very interested in Dr. Rey's concepts. He spent some time explaining methodology, at the level of the microscope, which will give observable slow and quick freezing effects on cells. I would like to ask that when water-binders, such as glycerol, are used the concentrations be mentioned and whether or not 1 stage or 2 stages of protected or unprotected freezing was involved.

Dr. Lasfargues: It has been recognized by various tissue culturists that the cultivation of the normal mammary gland is subject to some difficulties because of the fatty tissue embedding the epithelium. I tried to cultivate the mammary glands of mice after pregnancy, in the middle of pregnancy, or after parturition without special techniques. I got growth of epithelium in about 5 to 10 percent of the cases. I would like to ask Dr. Ranadive what is the percentage of growth of epithelium in her cultures.

Dr. Ranadive: I am not growing epithelium for long periods or on a large scale for cultivation of milk-borne virus as you are doing.

Dr. Lasfargues: You are growing normal mammary glands and you said you are getting epithelial growth. What is your percentage of success? How many times, in 100 cultures, do you get epithelium?

Dr. Ranadive: We are using Swiss females in mid-pregnancy. I have done about 12 experiments and I think we got 8 or 9 good epithelial cultures. We explant the tissue in the plasma clot on the glass wall of the tube. I do not use slips in tubes

because I find that I don't get good growth on them or on slides. But on the tube wall we get fairly good growth. Then we carefully pull out the plasma clot and stain the preparation on the slide. I don't think we find it difficult at mid-pregnancy. We find it difficult to get cultures from the virgin gland. Maybe it is just a coincidence but in 12 experiments of about 12 tubes each, I got epithelium in 50 percent of the tubes. That was done only to be able to compare the normal epithelial cells with the mammary-tumor epithelium that we were growing simultaneously. I was interested to find many abnormal cell divisions in mammary-gland epithelium. I don't know whether this has anything to do with the pregnancy or not.

Dr. Goldhaber: With regard to experimental oral carcinoma, if one applies a carcinogen (0.3 percent 3-methylcholanthrene in acetone) periodically to the mucosal and skin surfaces of the mouse lip, only tumors of the skin will develop. A possible explanation for the apparent resistance of the mouse oral mucosa is the lack of a portal of entry through the mucosa to the deeper-lying susceptible cells, as well as the possible action of the saliva in washing the carcinogen away. We have tried to test this hypothesis by means of fluorescence microscopy and have found that immediately after application, the carcinogen gets into the sebaceous glands of the skin, whereas on the oral-mucosa side only a trace of carcinogen can be found on the surface.

Dr. Ranadive: Yes, there is some trace on the surface. I have seen that too. There is a very thick, horny layer lining the cheek mucous membrane. Probably the substance is not absorbed in the cheek but is passed on to the internal viscera where lesions do appear. We are also testing the tobacco fractions on mouse skin. But do you mean to say that you could get frank epidermoid carcinoma with tobacco fractions?

Dr. Goldhaber: A very interesting clinical observation concerning buccal carcinoma in betel chewers was made by Balendra (Brit. Dent. J. 87: 83, 1949), who found that the chewing of betel (which contains tobacco) does not result *per se* in oral carcinoma. However, where betel chewers have sharp, jagged teeth, resulting in traumatic ulcers of the oral mucosa, these ulcers ultimately become cancerous.

Dr. Ranadive: There is some literature available on the clinical material but there is lack of extensive experimental data. We have published statistics from the hospital and have drawn similar conclusions. The groups investigated were divided in three: those who chew tobacco with lime, those who chew a mixture of betel nut, lime, and tobacco in Pān, and those who chew betel nut only. Among these three groups, it was observed that the percentage of cancer cases was higher in those who chew just tobacco with lime. That is why we have started working with tobacco. Of course, betel nut could be taken up later.

Dr. Earle: With the Tissue Bank of the National Naval Medical Center, Dr. Evans and her associates have been working now for some years trying to grow normal epithelium of the skin. We have now established a strain of such skin epithelium that has been maintained in culture for more than 3 years. It was taken originally from the normal skin of a 52-year-old man and is growing luxuriantly *in vitro*. Mr. Perry of the Tissue Bank, in collaboration with Dr. Sanford, recently established a clone of this strain of cells, and since some of you may have seen the clone picture of the original strain L-929 of mouse fibroblasts I thought you might like to see a photograph of the clone from human epithelium for comparison. (Figures not shown here; to compare these 2 figures *see* J. Nat. Cancer Inst. 18: 716–717, 1957.)

Captain Poley: I want to introduce one word of caution on the use of glycerine for freezing. Make sure that the product is as pure as possible. We have found that Eastman Kodak has a very good product, whereas others have a certain amount of impurity and toxicity.

Dr. Bang: I would like to ask Dr. Klein, in the light of Craigie's interest in the shape of the cell before it is frozen, whether the fact that he is working mainly with ascites cells has any significance in relation to their capacity to be frozen without glycerine and salt?

Dr. Klein: Not in our experience. We can use solid tumors, ascites tumors, and even leukemias equally well without glycerol. I think some of the impression that ascites tumors are more easily frozen than solid tumors may be due to the fact that a solid tumor is usually minced in salt solutions before freezing, whereas ascites tumors are

frozen directly. In our experience, neither a solid nor an ascites tumor will survive unprotected freezing if diluted with salt solutions.

Dr. Lasnitzki: We have also maintained a tumor bank at our laboratory for some time and found that addition of isotonic glucose to the tumor cell suspension before freezing increased the viability of the tumors.

Miss Owens: I should like to ask Dr. Klein about the growth curves of cells in ascites fluid. Does the amount of ascites fluid increase as the cells grow? What is the cell population per milliliter?

Dr. Klein: The cell population per milliliter depends on the tumor, the inoculum size, and time after inoculation. With the Ehrlich ascites tumor, a plateau of about 130×10^6 per ml. is reached soon after inoculation. With lymphomas, 200 to 400×10^6 per ml. are more usual values. The ascitic fluid increases as the cells multiply. The nature of the relationship between fluid and cells varies from tumor to tumor (*see* Révész, L., and Klein, G.: J. Nat. Cancer Inst. 15: 253–273, 1954).

The Future of Tissue Culture in Cancer Research [1, 2]

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Many aspects of the cancer problem already are being investigated by means of cells cultivated *in vitro*. Such studies include: assistance in the diagnosis and classification of tumors, the inhibitory properties of potentially chemotherapeutic drugs, and comparisons between normal and abnormal cell lines with respect to their physical interrelationships and competitions as well as their nutrition and metabolism. These investigations have been described or will be discussed by those more competent than myself. I need only note my confidence that because of this work new insight will be gained and new avenues of study will be opened.

Perhaps one of the fruitful ways in which an outsider can direct our thoughts toward the future of tissue culture in cancer research is to raise the following questions: To what extent is the art of cell cultivation now being employed to probe the basic theories concerning the origin and nature of the malignant cell? Which side of the tool should be sharpened if these theories and their implications are to be explored in more incisive fashion?

The distinctive merit of cultivating animal cells *in vitro* has long been recognized to be an analytical one. Dissatisfaction with the failure of tissue culture to contribute, as yet, notable illuminations of the cancer problem ignores the fact that pathology and biochemistry in a comparable state of immaturity were equally inadequate. The first step in the development of this newer tool necessarily has involved standardization of methods, of nutriment, and of environment to a point where cells could be maintained *in vitro* as objects for more penetrating study. With these obstacles largely cleared away, observations on the variability of cells are appearing constantly. In the future much more imagination can be employed in devising uncomplicated methods for studying cells under conditions which approximate more closely those of the cell community from which the isolates were derived. It is to be expected that much more attention will be given to duplication of *in vivo* gaseous atmospheres and feeding rates and to the interesting natural complexities attainable in the minute organ culture or artificial matrices (*1*).

[1] Received for publication May 28, 1957.

[2] Presented at the Decennial Tissue Culture Conference at Woodstock, Vermont, October 8-12, 1956.

Meanwhile, I would call your attention to the fact that present concepts concerning the origin and evolution of malignant cells seem susceptible to analysis and illumination by means of cells cultivated *in vitro*.

Mutation and Evolution

The theory of mutation seems adequate to explain all aspects of malignancy. There is no form of life in which the replication of genes can proceed without accident. It seems inevitable that the gateway to evolution should always open the bypaths of malignancy. If it is assumed that the earliest forms of metazoa arose from dissimilar clones that survived by compensating for mutual deficiencies and that increasing dependence and specialization resulted in the cell communities known as animals, we must never forget that we are nothing more than a collection of reproducible accidents. As microbiologists, we might think of this high degree of organization as being historically quite unnatural. We must expect that each cellular element will be captive to such systems only as long as it remains in a state of specialized dependence upon the other cells and components. In short, the general implications of the theory of evolution suggest that within the lifetime of any organized animal there may be produced millions of cells with simplified nutritional and metabolic needs and that among these only a small minority may be the progenitors of fully malignant clones. It is a special province of inquiry with cell isolates *in vitro* to determine the incidence, variety, and significance of these aberrations.

One of the possible approaches to these problems has become a vogue quite by accident. It will be recalled that a few years ago the *in vitro* maintenance of epithelial cells of adult origin seemed to be an impossibility. Today, the establishment of epithelial cell lines has become routine, perhaps primarily because of planting out original cell populations that number in the millions. The postulation that this revolution has occurred because of mutants among the explanted cells is consistent with reports of a general deterioration of cell sheets and their replacement by cells that arise from discrete centers of survival and growth.

Primarily explanted cells might serve as a background for plaque methods of analysis, in which each center of survival or growth is regarded as a clone. Since it should be practical to enumerate the incidence of successful centers of growth in comparison with the millions of cells explanted, the possibilities in such methods suggests a series of fascinating questions. Are there, at any stage in the history of such cultures, centers of surviving cells that present recognizable premalignant cytology? Would tissues from animals or persons with spontaneous carcinoma, or from those with such familial trends, yield a significantly higher ratio of *in vitro*-adaptable clones than tissues from presumably more stable sources? Or, on the contrary, would it turn out that the incidence of such cells within "normal" tissues is "normal" and actually not a determinant of the phenomenon of carcinogenesis?

Before passing to other considerations we must ask one further question.

What has become of the fabulous fibrocyte that used to preponderate in cultures from explanted tissues? Have we frightened this proliferative bully into assuming the guise of epithelial cells or simply lost him between the sheets? Or shall we insist that we are encountering the first evidence that wound healing and sarcomas are going out of style?

The type of problem posed thus far presupposes the explantation of large numbers of cells as primary isolates and the identification of different properties by means of what may be called "plaque" methods. One disregards theories concerning the *cause* of altered genetic constitution. One is concerned primarily with 2 items: *conditions* that favor early selection or recognition of cells that differ from the average by exhibiting greater independence of growth on selected minimal media and the association between these properties and spontaneous malignancy.

The Causes of Mutation

Great interest centers around the question whether malignancy arises most frequently from "natural" mutations or from mutations induced by extraneous agents, such as viruses, which might complicate the replication of genes. An oversimplified form of the virus theory might be summarized as follows: Certain viruses are known to cause tumors; new agents and new tumors of this type are capable of discovery; if this process continues, all malignancy may eventually be attributable to viral agents. The inadequacy of this theory as a generalization is obvious. It would deny that genetic accidents happen for other reasons and demand that the whole process of evolution and mutation be determined by one type of agency.

This type of theory, however, possesses one merit. It insists that the induction of genetic alteration by extraneous biological derivatives or entities requires consideration and that certain of these causes, when identified, might prove to be especially conducive to cancer.

Having taken the liberty of defining animals as a collection of reproducible accidents among protozoa, it may be permissible to take the future boldly in hand for a few moments in order to review what these mechanisms may be. I would first advise you that the science of microbiology was studied diligently for almost 70 years before any general concept of these factors was evolved and that my faith in the essential unity of biology will permit me to await whatever parallelisms may develop from the study of tissue cells.

Three major means for the biological sharing or induction of genetic character now seem to have been recognized, though no one of them is capable of demonstration within all bacterial species. These are: 1) gene recombinations in *Escherichia coli*, which are thought to have a sexual basis, in which intact bacterial cells are essential agents and by means of which several genetic markers may be exchanged concurrently (*2*); 2) genetic transformations in the pneumococcus and influenza bacilli, in which a single new character can be induced by the acquisition of deoxyribonucleic acid complexes derived by extraction of healthy cells

carrying the factor to be imposed (*3*); 3) viral transductions in staphylococci and paratyphoid and diphtheria bacilli, in which a single genetic character is transmitted by means of a latent bacteriophage (*4*).

Among recent examples of a carrier virus imparting a seemingly permanent character of virulence on its host cells, the diphtheria bacillus provides perhaps the most interesting observations. This organism has been the object of all manner of study for many years. The property of virulence or toxigenicity was thought to be inherent in the genetic make-up of such strains. Nevertheless, it has now been shown: that stable avirulent strains of *Corynebacterium diphtheriae* are transformed to fully virulent, toxigenic strains when converted to a lysogenic (*i.e.*, carrier) state by an appropriate bacteriophage (*5*); that the inducing capacity is limited to certain phages (*6*, *7*); and that loss of the carrier virus results in loss of virulence (*6*).

Thus, we observe that what appear to be constant, stable properties in a line of cells may be due to 2 dissociable biological entities.

Insofar as cancer research is concerned, several fortunate trends suggest that such lines of inquiry will not long remain closed to the student of tissue cells *in vitro*. In the first place, the patterns for the analysis of induced genetic differences are now rapidly being developed by microbiologists. In the second place, the art of cell cultivation has caught up with or surpassed the practices of the protozoologist, while the art of plating clones (*8*, *9*) has already been initiated. Though much work stands between vision and realization, the forest is rapidly being cleared away.

The Properties of Virulence

In an earlier section it was suggested that during the life of an animal millions of cells may exhibit modifications of nutritional requirement or metabolism which afford a basis for the property of malignancy. It was also questioned whether such modification alone will prove to be primary determinants of spontaneous cancers.

One of the fascinating problems for the future is a fusion between the prevailing concepts of the biochemist and the natural (scientific) suspicions of the biologist. By the very nature of his training and tools the biochemist must proceed on the assumption that a suitable combination of nutritional and metabolic properties may afford an explanation of malignancy. The biologist, meanwhile, is concerned about the probable occurrence within mutant cells of stereochemical modifications that serve as antigen determinants and also the presence or absence of host capacity to suppress cells carrying certain determinants. He wonders whether immunologic selectiveness in the host may not be a strait jacket that frequently overrides the nutritional and metabolic advantages possessed by certain types of mutant cells.

It will be the special privilege of the cellular biologist to separate lines of cells possessing different combinations of nutritional or metabolic modifications. He may be able then to ascertain whether the earliest

stages of cancer development depend in part upon host compatibility (insidiousness) of mutant cells and whether final malignancy depends primarily upon the completion of appropriate physiologic modification.

As an example of the study of virulence by instructive methods and material, the work of Sanford and associates (*10*) deserves close attention. Though the method of selecting 2 clones from among the descendants of a single fibrocyte from C3H mice was necessarily cumbersome at that time, 2 established clones have been shown to differ markedly in their virulence. Certain factors which contribute to these differences have been identified. The more malignant NCTC clone 2049 exhibits a greater capacity for anaerobic glycolysis, lower rates of oxygen consumption, and greater ability to incite a vascular response to nurture the implants in challenged animals. The less malignant NCTC clone 1742 produces a significant proportion of tumors only in X-irradiated mice. Whatever the impediments within this clone, it becomes fully virulent after a single mouse passage and exhibits the metabolic properties of a completely successful tumor.

Both clones and (the earlier) NCTC clone 929 of strain L cells induce an immune response in C3H mice. Since the low tumor clone becomes fully virulent during 1 animal passage, there has now been delineated an ideal circumstance in which to analyze further the immunologic and metabolic transformations that are essential to unrestrained growth in the original host.

Whether the attributes associated with the earliest stages in the development of cancer will be found among the clones of established cell lines or must be sought among cells explanted directly from tissues are questions that lend excitement to the future.

Recapitulation

In this limited discussion no reference has been made to the practical applications that may arise from broadening basic knowledge concerning the properties of malignant cells.

What has been said can be summarized briefly:

1) The cultivation of cells *in vitro* is a tool already partially developed and capable of playing a significant role in cancer research.

2) The problem of cancer is a problem of cellular mutants and their potentialities.

3) If cell and tissue cultivation are to afford tools appropriate to this type of problem, present skills in the management of cell lines must be supplemented with methods for determining the properties inherent in individual cells. It seems, therefore, that a major direction of development will consist in the art of plaquing, plating, manipulating, and analyzing the property of cell clones.

4) Happily, methods for genetic analysis are already highly developed and are being pushed forward. The bridges which

permit their application to animal cells appear to be in the process of sound construction.

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DISCUSSION

Dr. Murray: In the role of minor prophet, following a major prophet, I would like to lament that the office of soothsayer is fraught with difficulties, most especially in the field of science. If one's prophecy fails, one may provide free entertainment, but one gets no benefits from the comedians' union. If one is endowed with extrasensory perception and guesses right, at best one is diverting the cream from the coffee of some honest worker who grubs away and gets the actual results. But I must perforce follow Dr. Hanks into this angelophobe, imbecilotropic area.

This audience can certainly agree that the etiology of cancer must be investigated at the cellular level and that the cell *in vitro* offers unparalleled opportunity for analysis through direct observation and direct confrontation with experimental agents. As a basis for future etiological investigation along these lines, I would like to suggest the availability of hypotheses less orthodox than what used to be called the mutational theory, and more Lamarckian. At the present time, distinctions between these 2 approaches have largely broken down; consequently I may refer simply to data that do not concern the chromosomes. I would like to focus attention for a moment on cell transformations that have been effected by agents such as vitamin A, not known to be mutagenic, and on the so-called dependent cancers (as of breast and of prostate in man, and sex-spot in the hamster) whose incidence and course are greatly influenced by hormones. These were discussed in a stimulating way by Dr. Lasnitzki. Further, I would recommend to your attention the whole apparatus of induction as visualized by the experimental embryologists, from whom we have lately had such illuminating discussions. I use the term induction intentionally, invoking its broadest significance. I think one is within one's rights in deducing from these types of data that permanent or, at least, persistent transformations may be brought about by means other than random irreversible mutation and subsequent selection. The repeated observation of malignancies arising at the site of irritation by a great variety of agents, from tapeworm larvae to trauma, coupled with a long series of attempts to identify the special components of inflammatory exudates, make it appear not impossible eventually to align these malignant transformations with some principle of cytoplasmic or somatic induction. For direct investigation of such problems *in vitro*, the means are already at hand and a start has been made. These are among the most basic and the most urgent problems of neoplasia today.

The very great diversities among types of cancers that are observed by the pathologist, the morphologist, and the biochemist lend themselves well to diverse concepts of etiology. We all may be right, including those who espouse the now rapidly gaining theory of viral origin. Hardly anyone would disagree that this is not sufficient to account for all neoplasia. Nevertheless, it was long ago demonstrated that a number of animal tumors may be transmitted by cell-free tumor filtrates: the Rous sarcoma and the Shope papilloma have been mentioned here. Now in the Lucké frog carcinoma the combined observations of Fawcett and of Duryee—the latter *in vitro*—make it appear probable that the pathogenesis of this tumor is not a sudden mutation but a gradual progressive transformation from the normal renal tubule under the influence of viral products, whose first effects are observed in the nucleolus. The milk factor of mouse mammary carcinoma, which already has a ponderous bibliography, is now being subjected by Lasfargues and Moore to a similarly combined intensive study with tissue culture and electron microscopy.

The regular observation of normal-to-malignant transformation *in vitro*, begun with the chemical carcinogens in the 1940's, will undoubtedly continue in the 1950's as a crucial means of observing the action of other potential carcinogenic agents, not only viruses. We may not be using tobacco smoke or tobacco condensates on respira-

tory or bronchial epithelium, but we shall certainly be exposing these to more specific agents, obtained by fractionation. And we shall be testing hormones, their conjugates, and possibly their analogues upon the dependent human tumors, often with a direct practical relationship in view between the results from an individual biopsy and the clinical treatment of the patient.

Lately the research worker's imagination has been captured by the spontaneous regression of malignant tumors. The work of Sanford and her colleagues, cited by Dr. Earle, has demonstrated a decline in malignancy in clone strains from single cells. But regression occurs also in well-established and widely disseminated tumors, both animal and human, *in vivo*—the latter documented, for the human, in an article by Everson and Cole in the *Annals of Surgery*. This subsidence of tumor and metastases can hardly be explained by reversed mutation and it furnishes a problem that will receive increasing attention from the immunologist. The observations of Enders on partial cellular resistance to pathogenic agents may not inconceivably find application in studies of cancer.

It should not be forgotten that in regard to human tumors, tissue culture affords one of the few means of experimentation. Dr. Greene's admirable *in vivo* culture is another. Such methods are the bridge, however shaky, between animal experimentation and clinical trial. Where the individuality differential is so great and of such vital importance as in the spontaneous human malignancies, the culture of biopsy material for a host of tests—diagnostic, immunological, and chemotherapeutic—would seem, with the accumulation of knowledge and critical evaluation, to have a great and inevitable future.

I hope I may be forgiven this excursion into alien fields. I trust it may not seem more incongruous for me, as a cytologist, to predict a cancer immunology than for Dr. Hanks, as a microbiologist, to tee off on cancer. It simply proves once more that the grass looks greener on the other side of the fence!

Dr. Duryee: Experimental cancer research needs, and will continue to need, an increasing understanding of cellular physiology. Nuclear functions appear almost neglected, in contrast to those of the cytoplasm, which have been easier, as Dr. Hanks points out, for tissue culturists to modify and study. It may be useful to interpret some of the beautiful photographs presented at these meetings in terms of our experience with nuclear physiology as analyzed in cultures of amphibian renal adenocarcinomas.

Particular attention is called to a phenomenon, seen with difficulty in normal cells but enormously magnified in malignant ones, involving a pumping action or discontinuous output through the nuclear membrane. Essentially this results from nucleolar pulsation and discontinuous swelling of the nucleolar capsule, followed by bursting and extrusion into the cytoplasm. There is reason to believe that this is a normal method by which a ribonucleic acid (RNA) coacervate colloid is put out into the cytoplasm to serve later as a template for proteins. When one infects frog kidney tubules with a filtrate from a tumor containing a presumed virus, the first substance to appear is a new deoxyribonucleic acid (DNA) within the nucleolus of some host cells. New DNA can be followed in tissue cultures under phase microscopy by brilliant refractivity of the countable granules. This DNA could, of course, be the virus itself, but more probably it is an indication of protein associated with the virus. Thus the filterable agent can be shown to stimulate a fundamentally normal nuclear process to a very abnormal degree. We have found that the infected nucleolus may swell beyond the extent shown by Dr. Syverton and that the large inclusion bodies, which Dr. Lucké described in extreme and more malignant cases as filling the whole nucleus, are derived from nucleoli. In some instances, these may be retained within the nucleus, while in others they are extruded into the cytoplasm to make the typical kind of cytoplasmic inclusions that Dr. Weller and others showed us today. Following the appearance of new RNA, nephron cells increased in size, changed from cuboidal to columnar form, and lastly were stimulated to divide. Resulting hyperplasias could be graded in degrees of abnormal patterns all the way to those characteristic of true malignancy.

The fact that these processes can be followed exactly and related to abnormalities which other viruses cause in normal nucleolar physiology may be of value in formulating a unifying theory for the etiology of cancer. Of great importance is the idea that we can now pinpoint the nucleolar-chromosomal junction as a target for virus action. On this basis we may cautiously predict that organ-specific stimulants (such as the kidney virus) will be found for other cell types. Investigators should search their rapidly proliferating, monolayer tissue-culture preparations for unusual DNA granules in nucleoli. The question may well be asked: If viruses attack this specific genetic locus, can evidence be adduced for a related catalyst type of material synthesized at this locus? Future work should be directed toward finding whether such diverse agents as radiations, carcinogens, and viruses produce common intranuclear events leading to abnormal protein production.

Dr. deBruyn: So-called malignant growth is due to a change in the interaction between the cells and their environment. The environment consists of either cells, intercellular fluids, or gases. Much is known about the different causes of malignancy. The changes in the cells on their way to malignancy have been discussed in several ways. To discuss whether these changes are due to mutations, inductions, or transformations would take much longer than 8 minutes and has been discussed already. I think I had better postpone formulating a definition of what is really going on until more investigations have been done. Let us hope that in the next 10 years many results will be obtained. Cancer research depends largely on progress in other fields: experimental histology, embryology, pathology, biochemistry, etc., of what help tissue culture can be in these fields has been discussed by highly competent speakers during this stimulating and interesting Conference, which I am very glad to have been able to attend.

Because we are dealing with the interaction of the cell and its environment, it seems to me very profitable to work along the lines of research of Fell, Gaillard, and Wolff. Organ culture, in which tumor tissue is placed next to normal tissue, and the work of Grobstein, will give us, perhaps, more insight into the way in which the malignant cells invade their environment.

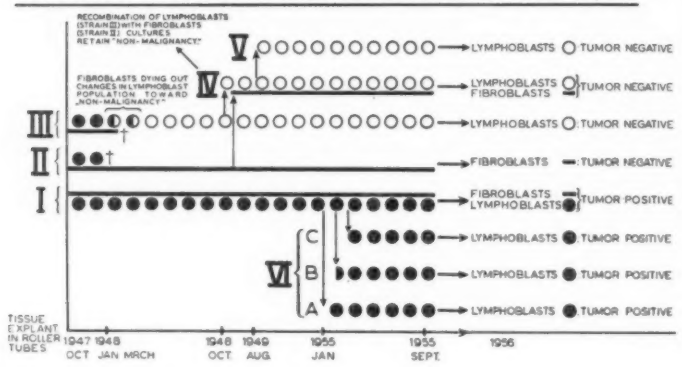
Much has been said during this Conference about the possibilities of cultivating large numbers of cells, of isolating single cells, and of studying the characteristics of the different clones obtained. This line of research will be very promising, because at present changes in the cells can be studied almost in the same way as done, with great success, with bacteria, molds, yeast cells, and noncellular organisms.

The effect of cancerogenic agents and of chemotherapeutic substances should be studied on strains of malignant cells and their normal prototypes *in vitro* and on transplantable tumors of the same kind *in vivo*. I will not go into this further but I would like to tell you something of what we have done and are doing in our laboratory at present in this line.

Years ago we intended to try to investigate leukemias and lymphosarcomas. At that time it was an open question whether these diseases could be considered malignant diseases or not. Figure 1, plate 76, shows a section of a tumor *in vivo*. It is a transplantable mouse lymphosarcoma which has many infiltrations in the spleen, the lymph nodes and the liver; shortly before death 40 percent of the leukocytes in the blood may be tumor cells—lymphoblasts. We cultivated this tumor and obtained good growth of both fibroblasts and lymphoblasts (fig. 2, plate 76). Both types of cells were multiplying; figure 3, plate 76, shows mitoses in the lymphoblasts and figure 2 shows a mitosis in a fibroblast. At that time not many of these tumors were grown. I could find out by separating these 2 types of cells and by using the micromanipulator that a few of the lymphoblasts, when injected into the peritoneal cavity of a susceptible mouse, could produce the tumor and kill the host within 16 days (*1*).

At the laboratory of Dr. Gey I developed the following strains (text-fig. 1) from one and the same subcutaneous tumor (*2*, *3*): a mixed strain (strain I)—fibroblasts and lymphoblasts (the lymphoblasts are tumor-positive); a strain that consists only of fibroblasts (strain II) and does not produce the tumor; and a strain (strain III, fig. 4, plate 76) in which changes in the cell population have taken place and which con-

CELL-STRAINS DERIVED FROM ONE SUBCUTANEOUS TUMOR OF TRANSPLANTABLE MOUSE LYMPHOSARCOMA MB (T 86157)



TEXT-FIGURE 1.

sists only of lymphoblastic cells that cannot produce the tumor. It was mentioned this morning that it might be possible to have malignant cells turn into nonmalignant, but we have to keep in mind that a selection of nonmalignant cells may have taken place from the beginning. I do not want to say that it is impossible that the change has taken place in the cells, but that it has been a selection of nonmalignant cells is not to be excluded.

The cells of strain I are growing in a kind of symbiosis and it was not possible to cultivate them without the mesenchyme. Last year, however, I succeeded in doing this by using a method other than the roller-tube method. We are cultivating the cells in slightly modified Erlenmeyer flasks (*4*) (fig. 5, plate 76).

Because the lymphoblasts are growing without mesenchyme cells, I was wondering whether in the long run they would also lose their "malignancy" as the cells of strain III probably have done. The malignant lymphoblasts have been cultivated, however, for more than 1½ years without mesenchyme and they still produce the tumor when injected into the peritoneal cavity of a susceptible mouse.

The cells in the ascites, produced by the injected malignant lymphoblasts, can be cultivated in Erlenmeyer flasks. The malignant lymphoblasts multiply *in vitro* and when injected into the peritoneal cavity of a mouse they produce ascites again. In this way we can go back and forth between the cells growing *in vitro* and *in vivo*. The strains described, therefore, are excellent material for studies of the morphology and the physiology of cells. Because the cells of these strains are growing in suspension they can be counted before and after the experiment. What the cells take out of the medium and what they add can be investigated.

Studies on the interaction between the different strains of cells, all derived from one and the same subcutaneous tumor, are in progress. We hope to get more information about the so-called loss of malignancy of the cells of strain III and about the difference between the non-tumor-producing and the tumor-producing cells. To make the picture complete, we maintain this lymphosarcoma also as a transplantable tumor in the mouse. The effect of chemotherapeutic and other substances is being studied *in vivo* and *in vitro*. I recently tried to maintain normal lymphoblasts in continuous culture. This is an exceedingly difficult job. I do not know if I may succeed.

I hope to have given you some idea of part of the work in progress in the Division for Experimental Cytology of the Netherlands Cancer Institute. The work may be briefly summarized as follows: Studies *in vivo* and *in vitro* of the characteristics of malignant cells and, where possible, of their normal prototypes, and the effect of

chemical substances on their growth. To achieve this aim several malignant and normal cell strains derived from mouse tissues have been developed. The tumor cells are maintained in continuous culture and as transplantable tumors in the mouse. Besides the strains of mouse cells several human cell strains are maintained *in vitro*.

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PLATE 76

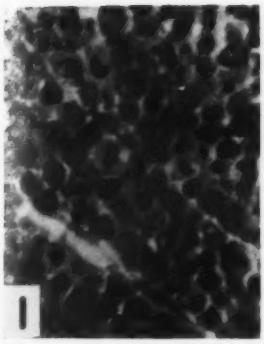
FIGURE 1.—Section of transplantable mouse lymphosarcoma MB (T 86157) *in vivo*. × 500

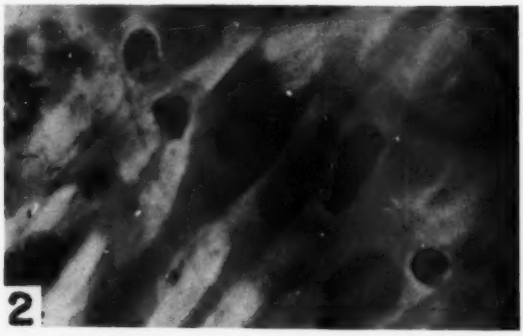
FIGURE 2.—Strain I, fixed and stained culture. Mitosis in a fibroblast. The cells have been maintained in continuous culture for a period of more than 5 years. × 900

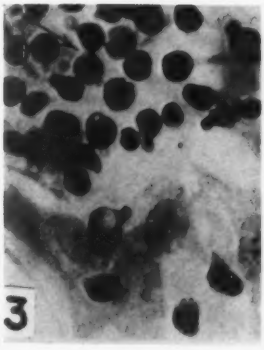
FIGURE 3.—Strain I, fixed and stained 3-day-old culture. Large mesenchyme cells, many lymphoblasts. Mitoses in the lymphoblasts (*arrow*). × 600

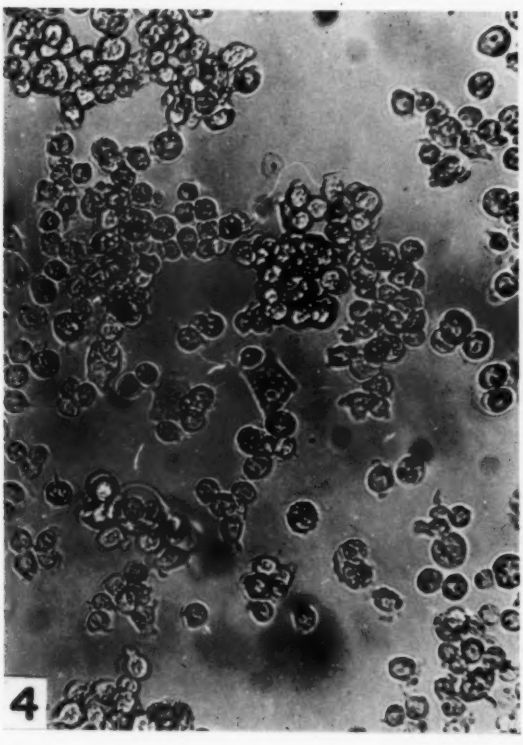
FIGURE 4.—Strain MB III, living culture, growing in Erlenmeyer flask. The cells do not produce a tumor. × 240

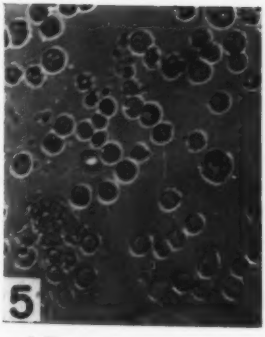
FIGURE 5.—Strain MB VI, living culture, growing in Erlenmeyer flask. The cells produce a tumor. × 240











deBruyn

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Dr. White: When I suggested future trends as part of our program, I am afraid I was misunderstood somewhat. I did not have in mind so much the matter of prophecy, which I know is extremely dangerous, but rather a little bit of planning. I think the most important results of the meeting that we had 10 years ago were the results of rather simple, broad plans. You are familiar with the objectives. Dr. Murray has carried through the preparation of the Bibliography brilliantly. It looked like an easy job; it proved to be a very hard job, but it has been accomplished and has proved worthwhile. Many of you are the products of the course in tissue-culture techniques which we laid out at that time. Dr. Parker, who is here, led the first course; it was subsequently taken over by Dr. Hanks, Dr. Merchant, and a number of others, and has been carried on by Dr. Murray and by some who, unfortunately, are not here. It has been instrumental to a considerable degree in making possible a meeting of this kind. We have representatives here of the third project, namely, the provision of a general, shall we say commercial, source of tissue-culture nutrients, glassware, and materials, means of simplifying the job for those of us who did not have either the time or the facilities to go into all of the niceties which appeared to be necessary for the profession and which we now know are in many cases quite unnecessary for the production of significant work. The fourth objective, the testing laboratory for these materials, has subsequently proved to be no longer necessary, since those who have taken over the job of preparing these materials have themselves established standards which we believe to be acceptable, under the constant supervision of some of our own people in an unofficial role.

Ten years ago we were able to lay out a series of rather simple objectives. That isn't possible now. One can no longer either predict or plan for a general approach. The best one can do is to look to the details. Perhaps I might note some of those directions which are not at the moment being adequately covered. That is what I have had in mind in listening to these programs, over the last few days—to see if there were any things which might be stressed more than they have been.

I have noted 3 such lacunae. They are not complete lacunae because all 3 have been mentioned, but they have been touched on less than I would have thought likely. One of these is the matter of single-cell isolations, how they are to be done, and what is to be done with them. I should like to devote a moment to that. Of course we have Dr. Evans' and Dr. Earle's and Dr. Sanford's method of isolating clones in capillary tubes, which is eminently successful but involves such complications that it cannot be done as a mass procedure. It seems to me that both on the animal and plant side what we need is a method by which we can isolate not 9 clones and discard 7 of them, but by which we can isolate 100 clones today and carry them all and then a week later isolate another 100 and a week later isolate another 100. That is not necessarily quite so big a job as it sounds, if this method of Puck's is as successful as it appears to be and if we do not attempt to permit each one of those clones to fan out into a very broad spectrum but follow it as a single, relatively narrow line. In my own laboratory, with plant cultures, for example, we maintain 20 cultures of each of about a dozen strains. At any time we can build one of these up into a broad spectrum, but for source purposes only, 20 is quite sufficient. It seems to me what is necessary in studying this thing in both our plants and animals is to isolate a series of these clones and follow them for considerable periods, because, as I have told a number of my colleagues, one of the things that I would like to see done is to determine whether these differences that we find in the clones (and everybody recognizes their presence) arise by a separation from a series of types of cells already there, or by mutations, or by a gradual adaptation and a gradual change. These are essentially the 3 theories of the origin of cancer. We should isolate a series of 100 such strains and compare them either nutritionally or by malignancy tests, by morphology or any other means. if what we have done is to select variants already present, we should get a series of variant strains right at the start, and in the second selection we should get relatively few such varied strains. If we are dealing with a case of mutation, we should have a constant level of incidence of these new variant strains. If, on the other hand, we are dealing with the occurrence of adaptation we should have a constantly increasing

incidence of these variant strains. I think that such a method, if applied on a mass scale, should give us a measure of which of these is significant. Perhaps all of them are, but we would like to know in what percentage they are important.

A second thing which I have heard mentioned briefly at these meetings, but which I think needs much more attention, is the matter of the temperature sensitivity of many of these responses. I know that Dr. Braun, for example, could have told you a great deal about the temperature control of the production of crown-gall tumors. He didn't enlarge on this subject to the degree that I expected. That is a response that is sensitive to a change of about 5 degrees and in which the critical period for that temperature influence begins with the 30th hour after the beginning of the culture and ends with about the 36th hour. It is very closely delimited both in temperature and in time. I would like to know whether many of these phenomena about which we have talked might not be similarly sensitive. We know, for example, that with our spruce cultures, one of the things which gives us a tumor in spruce trees is the fact that the cells are growing in the tumor for about 9 months out of the year, whereas in the normal tree they are growing only about 2 months in the year. There is a very definite time scale there. The question immediately arises: Is that because the normal tissue grows only at the high, summer temperatures whereas the tumor tissue has a wider temperature range and can grow at lower, autumn temperatures?

Still a third problem that I should like to see fully investigated arises from an examination of what Dr. Gaillard and Dr. Moscona and some of the others have mentioned. It comes to mind in view of what Dr. Kehr spoke of this morning. I should like to see some of these Graafian follicles that Dr. Gaillard has grown carried a little further to see whether we can get some eggs that can be fertilized; because it seems to me that one might study the genetics of changes that way. One of our standard techniques in the Jackson Laboratory is the transplantation of ovaries into castrated animals and having the animals fertilized. We can get 4 fertile females out of 1, because 1 ovary can be divided and transplanted into 2 animals and the female has 2 ovaries to be divided, so we get 4 good mothers out of 1 bad one. I don't know whether you, Dr. Gaillard, have tried putting any of these ovaries into castrated mice and then having those mice fertilized. It seems to me that it is a perfectly possible technique and corresponds in a way to the thing that Dr. Kehr talked about—tobacco plants treated with colchicine to produce the doubling of the chromosome number and hence a return of fertility.

These are just 3 things which have occurred to me in listening to the various talks here. I think there are a lot of things that we still can do.

Dr. Brakke: There have been some interesting preliminary reports on the transplantation of nuclei between amoebae to determine whether certain properties are under nuclear or cytoplasmic control. I wonder if this technique might show whether the malignancy of certain cell lines is nuclear or cytoplasmic.

Dr. Lumsden: In regard to the question whether changes can occur at a fundamental genetic level in the somatic cell in tissue cultures, I should like to suggest that it might be worthwhile examining for isoagglutinogens in human cells from old strains of many years' maintenance in culture conditions. It would be interesting to see if there is any loss of isoagglutinogen content or pattern in cells which have dedifferentiated during prolonged culture. Human cells would be the most suitable type since the original blood group of the patient is likely to be available in reliable hospital records from the time the specimen was obtained which was used as the source of the culture strain. This leads me to the (not disinterested!) question as to what are the oldest strains of normal human cells presently available.

Dr. Gey: The only long-term examples of stable normal strains are the ones derived from the rat that we have in the laboratory. These have been under cultivation continuously since 1938. Some of the normal strains, for example, the stable normal-rat strain 14p, have been derived from one of these, both in our laboratory and in other laboratories. Our derivative was the original malignant strain T-333. The stability of tumor-cell strains is also a subject of great interest. We have maintained a stable strain of the Walker rat sarcoma 319 in continuous culture for a quarter of

a century. This strain, even after growing in heterologous media for this long time, has retained its cultural and cytological characteristics and still produces a tumor in the rat. Other normal and tumor-cell strains have, as we all know, changed during cultivation. I am sure that Dr. Earle, who has talked about this here, will back me up in saying that we must qualify our statements with regard to the designation "normal" strain and also what we mean by "stability" in a strain.

We have made a bold effort to evaluate the various changes that occur in the human cervix which eventually lead to malignancy. Here one has a wonderful opportunity to get any number of specimens. Presumably one can follow what is conceived to be a series of developmental stages from the normal, altered under the influence of changes in age and hormonal background and so forth, through the stages of basal-cell hyperactivity, of intra-epithelial replacement, and finally to early invasive carcinoma and the autonomous, highly invasive and metastasizing tumor. In the first category we have seen no clear-cut examples of eventual rapid proliferation in tissue cultures of the normal and the basal-cell hyperactivity stage or in the intra-epithelial (carcinoma *in situ*) stages. We have made an effort, especially, to get stable strains established from the normal. When, however, we harvest from the category of early invasive or metastasizing tumors we do get cell strains that proliferate as continuous cultures. Here, however, the strains are not alike. For example, our HeLa strain, which has now been grown for over 5 years, is quite different from another tumor strain ElCu which consistently grows slower in culture, yet was derived from a cancer which still killed the patient. These differences in the behavior of the various cells derived from the same tissue need to be resolved, perhaps through tissue cultivation, and under conditions which will permit us to understand the developmental stages of this malignancy.

Dr. Earle: Over a period of years we have seen in tissue culture a number of cell strains which by ordinary criteria would have been classified as nonmalignant. In our laboratory, when we have established such a strain, we have tried to test it exhaustively. In every instance in which we have been able to do this, we have been able to break the nonmalignancy reputation of that strain—by demonstrating its ability to give rise to tumors which ultimately caused the death of the host, and which on section had a characteristic morphology. The strain L cells, for instance, originally were able to give rise to sarcoma in more than 66 percent of the C3H mice injected with them. These cells, gradually drifted down over a period of years to where we were unable to originate any tumors from them for quite a long time. Finally, we thought this such a critical point that we concentrated on it and did get one sarcoma from a large number of injections. By the use of heavily irradiated mice we got a substantial number of sarcomas. We no longer consider this cell strain as a normal strain in the sense of being entirely nonmalignant. I don't think that it ever has been since we first originated it and demonstrated its high malignancy.

The question that disturbs me is, however, what are our real criteria on which we can rely to establish the nonmalignancy of a cell line? I consider this is one of the critical areas of research that should be called to the attention of this organization as needing much further study. I don't know of any strain of cells in our own laboratory except the human skin epithelium strain which can really be considered nonmalignant, and I wonder whether there exists one in any other laboratory. What the ultimate fate of this human skin epithelium will be I will leave to the passage of time and to future research. However, in going to the use of heavily irradiated animals we are going to artificial extremes in trying to demonstrate malignancy. The cells of strain L are able to produce so small a percentage of tumors arising from injection of its cells into mice of its strain of origin, that I am strongly inclined to think of it as "clinically" nonmalignant. I would pose the question for your consideration as to whether at some point our criteria do not exceed, let us say, the demonstration of what I would, for want of a better term, call "practical clinical" malignancy and get into a completely alien and artificial field, even though it is a tremendously absorbing and interesting field from an experimental point of view.

One other point may be of interest. In our own carcinogenesis research, we treated a strain of mouse fibroblasts with a carcinogen, methylcholanthrene (MCA) in graded doses. The most heavily dosed cells were subjected to the agent for 406 days. At the end of that time we got severe alterations in the treated cells; the degree of alteration in any cell line was definitely directly correlated with the dosage of the carcinogen and was reflected both in changed cell morphology and behavior. That strain treated for 406 days was finally taken out of the carcinogen because the cells were so tremendously altered that while mitosis was extremely rapid it was almost or completely compensated by necrosis. The rate of growth of the cultures had dropped to practically zero, and it was most difficult to ultimately get the cell strain to survive. In other words, by altering these cells we had carried them so far beyond that stage of optimal cell transformation that, when we tried to inject animals with these cells they were rarely able to give rise to sarcomas; actually less than 1 percent of the injected mice showed tumors; usually the injections gave no growth. If only a limited group of injections had been made the cells would have been considered nonmalignant. I would call your attention to the probability that in progressive cell transformation there is not only a demonstrable malignancy range of cell alteration, but there is probably also an extreme range of cell alterations and transformations which to all intents and purposes are beyond the range of demonstration of malignancy by injection into normal hosts. In this range, which I find it useful to think of as a "supra malignant range," the cells are so altered that apparently they can hardly live either *in vivo* or *in vitro*.

Dr. Leighton: Dr. Earle, I want to ask you a question about your low and high tumor-producing sublines about which you spoke earlier in the day. Have you selected for further study the animals that failed to get tumors after inoculation with either subline? Is there any evidence that mice that do not get the tumors will subsequently develop tumors if exposed to cortisone or X ray? Should negatives become positives on exposure to cortisone or X ray, one might have a picture of great interest. The response of such latent tumors to chemotherapy might be qualitatively different from that observed in rapidly growing transplantable tumors.

Dr. Merchant: An analogy might be made between malignancy of a cell strain and virulence of a bacterium. In discussing virulence of a microorganism we must be prepared to discuss also the resistance of the host and to consider the interplay of host resistance and virulence of the parasite in order to determine whether infection will occur in any given instance. Thus in determining the ability of a cell strain, which has been maintained *in vitro*, to produce tumors in a given host many factors concerning the resistance or susceptibility of the host must be considered.

Dr. Harris: Dr. Brues, did you wish to comment?

Dr. Brues: I don't think we want anyone to go home believing that we all have confidence in mutation theory, and I will give you just one statement which is based on facts that you are all familiar with. The human being and the mouse develop about the same number of spontaneous tumors per organism. This means the mouse has about 1,000 times as many per cell. The two are irradiated and you find the same thing only even more cogently to the mutation question. So any mutational equation has to have a little fine print put in it; and this fine print, I think, is going to be a function of the total organism. The same goes for any theory, which says blindly that cancer is a disease of the cell and that one can forget the rest.

Dr. Klein: While I certainly don't disagree with what Dr. Brues has said, I was going to comment on the question whether the changes were reversible or irreversible. They seem to be irreversible at the population level. At the cellular level we don't know, because we do not have any system in which a reverse change would be detectable. As long as there are unchanged cells in the population, these have a selective advantage and no matter how many cells change in the reverse direction they remain undetectable in our present systems because they are at a disadvantage. They would only be detected by some kind of clonal analysis of the type that Dr. White was suggesting.

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